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From the Department of Pharmacology and Department of Chemistry Karolinska Institutet Stockholm Sweden

Biological Effects of an Unsaturated Trihydroxy Acid ($PGF_{2\alpha}$) from Normal Swine Lung

Prostaglandin and Related Factors 13

By

Erik Änggård and Sune Bergström

Received 6 September 1962

Abstract

Änggård E and S Bergström *Biological effects of an unsaturated trihydroxy acid ($PGF_{2\alpha}$) isolated from normal swine lung* Acta physiol scand 1963 *58* 1—12 — The effects on various isolated smooth muscle organs and on the cardiovascular system of cats and rabbits were studied The rabbit duodenum guinea pig ileum rat duodenum and colon hen rectal caecum guinea pig uterus and rat uterus were stimulated to a slow contraction Rabbit duodenum and oestrogen treated rat uterus proved the most sensitive organs with a threshold concentration as low as 0 001 μg/ml I v injection in rabbits of 15—30 μg/kg caused a fall in blood pressure with no changes in right ventricular pressure or heart rate I v injection of 15—30 μg/kg in cats increased right ventricular pressure with a concomitant depression of the systemic blood pressure A bradycardia which could be abolished by vagotomy or atropine occurred 10—30 seconds later It was concluded that in cats the fall in blood pressure resulted partly from a reduced cardiac output due to constriction of the pulmonary vessels partly from the bradycardia and to a minor extent only from direct effects on muscle vessels

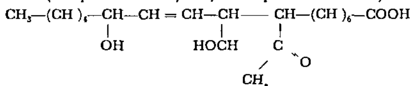
The smooth muscle stimulating activity of the prostaglandin present in seminal plasma of man and sheep and in extracts of the vesicular gland of sheep (Goldblatt 1933 Euler 1934 1939) has recently been found to be due to several related compounds The compound first isolated in pure form from vesicular glands of sheep PGE_1 has been found to have the structure

shown below (I) and recently two related compounds were isolated, PGE_2 and PGE_3 that contain respectively one and two additional double bonds (BERGSTROM et al 1962 a, b)

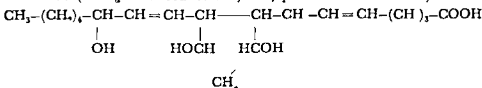
These authors also observed smooth muscle stimulating activity in the acidic fraction of the lipid extract from normal lungs of sheep and pigs The purification of these factors has led to the isolation of the same new compound from both sources which is related to the prostaglandins mentioned above and in the following When the different prostaglandins PGE_1 PGE_2 and PGE_3 are reduced with sodium borohydride each yields two isomeric trihydroxy compounds (cf BERGSTROM et al 1962 b) designated $PGF_{x\alpha}$ and $PGF_{x\beta}$[1] of which the former isomer in each case had slightly higher smooth muscle stimulating potency on rabbit duodenum than the parent compound and the latter considerably less It has been found that the factor isolated from normal lungs is identical with the more active of the two reduction products of PGE_2 and can thus in this nomenclature be called $PGF_{2\alpha}$ with the structure shown below (II) (BERGSTROM et al 1962 c)

The studies of the biological properties of the factor isolated from swine lungs were started with concentrates before the pure compound was available but the effects reported here are all due to this factor alone

I (PGE_1 = 11α 15 dihydroxy 9 keto prost 13 enoic acid)



II ($PGF_{2\alpha}$ = 9α 11α 15-trihydroxy prosta 5 13 dienoic acid)



Methods

The acid was prepared as described by BERGSTROM et al (1962 c) Samples of the preparation were weighed and dissolved in 0 15 M phosphate buffer (pH 6 9 or 7 4) and kept at —20 C until tested

The isolated organs were suspended in a bath of controlled temperature oxygenated with 6.5 % CO_2 in O_2 The movements were recorded isotonically on a smoked drum with a linear frontal writing lever

[1] The subindex x (x = 1 2 or 3) refers to the parent E-compound.

Solutions of the following composition were used

Tyrode solution NaCl 0 8 per cent, KCl 0 02 per cent $CaCl_2$ 0 02 per cent, $MgCl_2$ 0 0093 per cent $NaHCO_3$ 0 1 per cent NaH_2PO_4 0 004 per cent glucose 0 1 per cent

De Jalon s solution. NaCl 0 9 per cent, KCl 0 042 per cent $CaCl_2$ 0 006 per cent $NaHCO_3$ 0 05 per cent glucose 0 05 per cent

Krebs Henseleit solution NaCl 0 69 per cent KCl 0 035 per cent $CaCl_2$ 0 028 per cent, $MgSO_4$ 0 011 per cent, $NaHCO_3$ 0.21 per cent, KH_2PO_4 0 014 per cent glucose 0 1 per cent

Atropine 1.5 10^{-6} M and mepyramine 2 5 10^{-7} M were added in some experiments on isolated intestines but since these additives did not alter the responses they were omitted in the majority of the tests

Particulars for the various organs were as follows *Rabbit duodenum* Animals killed with intravenous air or blow on the head 2—3 cm strip of duodenum close to pylorus Tyrode solution 10 ml bath temp 37 C

Guinea pig ileum Animals killed by blow on the head and bled to death 2 cm segment from distal ileum Tyrode solution 4 ml bath temp 37 C

Rat duodenum Animals lightly anesthetized with ether and then bled to death 2 cm strip close to pylorus De Jalon s solution 4 ml bath temp 37 C

Rat colon Animals lightly anesthetized with ether and then bled to death 2 cm segment from descending colon de Jalon s solution 4 ml bath, temp 37 C

Cat duodenum Cats 1 4—2 6 kg anesthetized with ether and then bled to death 2—3 cm of proximal duodenum Tyrode solution 10 ml bath, temp 37 C

Hen rectal caecum Young hens were killed by cutting the neck 3—4 cm segment of rectal caecum s narrow proximal end Tyrode solution 10 ml bath temp 37 C

Rat uterus Virgin animals 100—200 g were lightly anesthetized with ether then bled to death In 5 experiments 1 mg estradiolbenzoate (Follidrin C benzoat, Astra) was given i m 24—48 hours earlier De Jalon s solution atropine 1 5 10^{-7} M 4 ml bath temp 32 C

Guinea pig uterus Non pregnant animals 280—480 g were stunned by a blow on the head and bled to death 2—3 cm strip of the uterine horn De Jalon s solution atropine 1 5 10^{-7} M 4 ml bath temp 32—35 C

Tracheal chain preparation The cartilagenous sections of tracheal rings from cats rabbits and guinea pigs were removed and 5—6 segments were tied together with fine nylon thread as described by AKCASU (1959) Krebs Henseleit solution lightly balanced balsa wood lever 4 or 10 ml bath temp 37 C

Bronchial chain preparation Intrapulmonary bronchi from cats were carefully dissected out and cut into rings (HAWKINS and SCHILD 1951) and were tied together with fine nylon thread and suspended in a 4 or 10 ml bath Krebs-Henseleit solution temp 37 C.

In vivo experiments were performed on cats weighing 1 1—3 8 kg anesthetized with chloralose (25—50 mg/kg i v) and urethane (0 2—0 9 g/kg i v) and on rabbits weighing 2 3—3 1 kg anesthetized with urethane (1—1 6 g/kg i v) The trachea was cannulated Arterial pressure was recorded in one of the carotids by a Statham pressure transducer (P 23 AA) Heart rate was determined by means of an interval recorder (GOLDSCHMIDT and LINDGREN 1962) via impulses from the blood pressure channel of the Grass instrument. Rectal temperature was maintained at about 37 C by means of a heating lamp directed towards the animal s chest I v injections were made through a plastic cannula inserted in the right jugular vein, with its tip a few cm from the heart

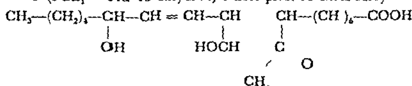
Right intraventricular pressure was recorded with a Statham pressure transducer (P 23 AA) Following thoracotomy and positive pressure artificial respiration a polyethylene catheter was advanced through the ventricular wall into the cavity with the aid of a sharp metal probe which was then removed. Injections in the left ventricle were

shown below (I) and recently two related compounds were isolated, PGE_2 and PGE_3 that contain respectively one and two additional double bonds (BERGSTROM et al 1962 a b)

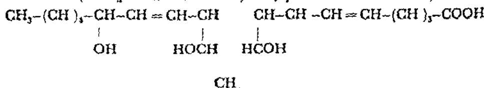
These authors also observed smooth muscle stimulating activity in the acidic fraction of the lipid extract from normal lungs of sheep and pigs The purification of these factors has led to the isolation of the same new compound from both sources which is related to the prostaglandins mentioned above and in the following When the different prostaglandins PGE_1 PGE_2 and PGE_3 are reduced with sodium borohydride each yields two isomeric trihydroxy compounds (cf BERGSTROM et al 1962 b) designated $PGF_{x\alpha}$ and $PGF_{x\beta}$[1] of which the former isomer in each case had slightly higher smooth muscle stimulating potency on rabbit duodenum than the parent compound and the latter considerably less It has been found that the factor isolated from normal lungs is identical with the more active of the two reduction products of PGE and can thus in this nomenclature be called PGF_{α} with the structure shown below (II) (BERGSTRÖM et al 1962 c)

The studies of the biological properties of the factor isolated from swine lungs were started with concentrates before the pure compound was available but the effects reported here are all due to this factor alone

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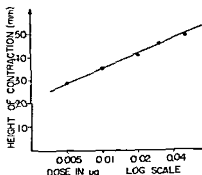


Fig 2 Dose response regression for PGF on isolated rabbit duodenum The responses shown in Fig 1 are plotted against the log dose Note linear regression curve

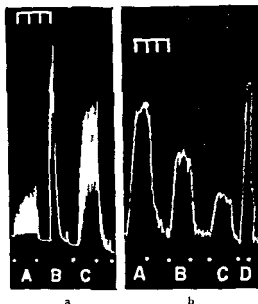


Fig 3a Isolated duodenum cat. Bath 10 ml Tyrode solution Time in minutes
A = 0 1 µg PGF2α
B = 0 3 µg acetylcholine
C = 1 µg PGF2α

b Isolated duodenum, rat Bath 4 ml De Jalon s solution Time in minutes
A = 0.21 µg PGF2α
B = 0 14 µg PGF2α
C = 0 07 µg PGF2α
D = 0 5 µg acetylcholine

Results

Rabbit duodenum

This preparation is extremely sensitive to PGF The contraction starts after a latency of 10—20 sec and slowly proceeds to a maximum within 2 min The spontaneous pendulum movements typically increase in amplitude but may remain unchanged Relaxation is also slow and takes 1—3 min (Fig 1) Reproducible quantitative effects with a straight log dose response relationship were obtained in concentrations from 0 001 µg/ml (Fig 2) but even smaller doses produced a definite contraction

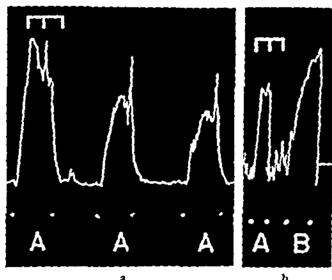


Fig 4a Isolated ileum guinea pig Bath 4 ml Tyrode solution with atropine 1 5 10^{-6} M and mepyramine 10^{-7} M Time in minutes Note tendency to tachyphylaxis.
A = 1 µg $PGF_{2\alpha}$
b Isolated rectal caecum hen Bath 10 ml Tyrode solution Time in minutes
A = 0 1 µg acetylcholine
B = 0.5 µg PGF

Cat duodenum

$PGF_{2\alpha}$ stimulated this organ to a contraction and increased spontaneous activity (Fig 3a) in concentrations from 0 01 µg/ml As in the guinea pig ileum decreasing sensitivity to $PGF_{2\alpha}$ was observed on subsequent stimulations

Rat duodenum

PGF stimulated this organ in concentrations from 0 025 µg/ml and provoked increased spontaneous activity (Fig 3b) No tachyphylaxis was noted

Rat colon

In 2 experiments $PGF_{2\alpha}$ produced a contraction in concentrations from 0 125 µg/ml

Guinea pig ileum

$PGF_{2\alpha}$ in concentrations from 0 025 µg/ml caused a slow contraction starting after a latency of 5—15 sec Varying degrees of tachyphylaxis were always noted as was a tendency to develop irregular spontaneous activity (Fig 4a) Subsequent histamine induced contractions were potentiated

Hen rectal caecum

This preparation was very sensitive to $PGF_{2\alpha}$ Concentrations from 0 005 µg/ml produced a slow contraction and increased spontaneous activity (Fig 4b), continuing several minutes after the bath solution was changed

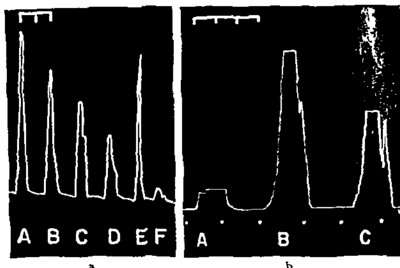


Fig 5a Isolated uterus from virgin rat Bath 4 ml De Jalon s solution with atropine 1 5 10- M Time in minutes
A = 0 1 μg PGF α
B = 0 08 μg PGF₂α
C = 0 06 μg PGF₂
D = 0 04 μg PGF₂α
E = 0 1 μg 5 HT
F = 0 08 μg 5-HT
Note diff rent dose response relations for PGF₂α and 5 HT

b Isolated uterus from nonpregnant guinea pig Bath 10 ml De Jalon s solution with atropine 1.5 10^{-7} M. Time in minutes
A = 0 1 μg PGF α
B = 0 ? μg PGF₂α
C = 0 15 μg PGF α

Rat uterus

A strong stimulatory effect was noted in concentrations from 0 01 μg/ml (Fig 5a) Pre treatment of the rat with oestrogen increased the sensitivity of the uterus 10—50 times Lysergic acid diethylamide in 0 1 μg/ml had no effect on PGF but blocked equi-effective doses of 5 hydroxytryptamine (5 HT) completely

Guinea pig uterus

PGF caused this organ to contract in concentrations from 0 01 μg/ml (Fig 5b)

Tracheal chain preparation

The responses of tracheal muscle from cat rabbit and guinea pig were weak even at concentrations up to 2 5 μg/ml In one preparation from a guinea pig however a strong reproducible stimulatory effect was noted at a concentration of 0 5 μg/ml in the bath

Table I Approximate threshold doses on various test preparations for $PGF_{2\alpha}$ and comparison of activity with $PGF_{1\alpha}$

Test preparation	Threshold dose in μg per ml bath volume	
	$PGF_{2\alpha}$ (present investigation)	$PGF_{1\alpha}$ (Bergström et al 1959)
Rabbit duodenum	0.001	[1]0.003–0.01
Cat duodenum	0.01 –0.05	—
Rat duodenum	0.025–0.1	[1]0.08 –0.3
Rat colon	0.125–0.5	—
Guinea pig ileum	0.025–0.1	0.3 –1
Hen rectal caecum	0.005–0.01	0.01
Rat uterus	0.01 –0.05	0.05
Rat uterus, estrogen treated	0.001	—
Guinea pig uterus	0.01 –0.02	0.2

[1] Jejunum used instead of duodenum

Bronchial chain preparation

In two experiments on bronchi from cats $PGF_{2\alpha}$ in 0.1—2.5 μg/ml was without effect although the chain showed high sensitivity to acetylcholin

Table I gives the approximate threshold doses on various test preparations and compares the activity of $PGF_{2\alpha}$ and $PGF_{1\alpha}$ (Bergström et al 1959) $PGF_{2\alpha}$ appears in general to be slightly more active than $PGF_{1\alpha}$

Circulatory and respiratory system

Cats

Intravenous injection in cats of 15—30 μg/kg increased right ventricular pressure within a few seconds with a concomitant fall in systemic blood pressure 10—30 sec later there occurred a bradycardia which could be more or less pronounced and in a few experiments was entirely absent The blood pressure tracing often had a biphasic appearance the first fall being associated with the increase in right ventricular pressure the second and slower fall with the onset of bradycardia Vagotomy or atropinization abolished the bradycardia and reduced the intensity and duration of the blood pressure depression to a variable extent the rise in right ventricular pressure remaining unchanged (Fig 6) If initially the bradycardia was pronounced vagotomy or atropine were more effective in antagonizing the depressor response of $PGF_{2\alpha}$ The quantitative relationship between the various factors responsible for the fall in blood pressure proved difficult to establish owing to a tendency to tachyphylaxis

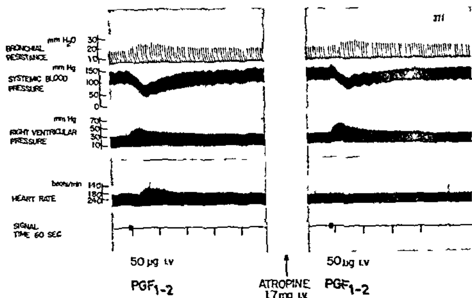


Fig 6 Effects of i v inje tion of PGF $_{\alpha}$ before and after atropine Cat 2 1 kg Chloralose 100 mg urethane 0 6 g Note that the bradycard a occurs after about 20 sec lat ncy and that it is abolished by atropine

Injection into the left ventricle caused no or little immediate fall in blood pressure After a latency of 7—15 sec the same effects but less pronounced were observed as after i v injection indicating that the substance had recirculated

The direct effects of PGF$_{\alpha}$ on vascular beds were also studied in muscle intestine and kidney Intraarterial injections of 1—10 µg into a muscle moderately increased the flow of blood through that area indicating a vasodilation Injected into the superior mesenteric artery 1—10 µg of PGF$_{\alpha}$ produced a slight decrease in blood flow This effect could however be secondary to contractions of the smooth muscle layers of the intestinal wall I a injections of 1—10 µg into the kidney did not affect blood flow through that area

The fall in blood pressure could also be partly explained by reflex inhibition of vasoconstrictor tone in peripheral vessels In 3 experiments the cross circulated hind leg of a cat showed no increase in blood flow when PGF$_{\alpha}$ was injected i v into the recipient animal On the contrary the blood flow decreased to a varying degree denoting vasoconstriction This effect was possibly of baroceptor reflex origin

An increased bronchial resistance was regularly observed following i v injection of 15—30 µg PGF (Fig 6) This effect could be due to constriction of the bronchial smooth muscles or to displacement of air into the recording system, secondary to pulmonary congestion or both

Rabbits

$PGF_{1\alpha}$ injected i v in 15—30 μg/kg caused a fall in systemic blood pressure lasting 3—10 min, depending on the dose No effects were observed on right ventricular pressure or heart rate Owing to the limited supply of pure $PGF_{1\alpha}$ the effects were not further analyzed

Discussion

The factor isolated from swine lung ($PGF_{2\alpha}$ (II)) was found to be a highly potent smooth muscle stimulating substance with a wide range of biological activity Its effect when added to an isolated smooth muscle organ is to produce a slow contraction The most sensitive organs tested were the rabbit duodenum and the estrogen treated rat uterus both of which responded to concentrations as low as 0 001 μg/ml Varying degrees of tachyphylaxis were noted on the guinea pig ileum and the cat duodenum Obviously this makes these organs unsuitable for the assay of $PGF_{2\alpha}$

Of the organs tested the rabbit duodenum seemed to be particularly well suited for routine assay as it is highly sensitive and gives quantitatively regular responses for several hours

The effects of $PGF_{2\alpha}$ on isolated tracheal and bronchial muscles from cat rabbit and guinea pig were weak On the other hand, an increased bronchial resistance was consistently seen upon i v injections in cats Admittedly this could be secondary to circulatory effects of $PGF_{2\alpha}$ as discussed on p 9 Further analysis of this effect is necessary to establish if $PGF_{2\alpha}$ has any broncho-constrictor effect *in vivo*

Intravenous injection in cats of 15—30 μg/kg caused a marked rise of right ventricular pressure with a fall in systemic blood pressure and somewhat later a bradycardia As the rise of pressure in the right ventricle occurred concomitantly with the fall in blood pressure it seems possible to assume that the first phase of the depression resulted from a decreased cardiac output due to constriction of the pulmonary vessels The bradycardia appeared with a latency of 10—30 sec and could be abolished by vagotomy or atropinization The decrease in heart rate could then be a reflex effect elicited from pressure receptors in the lung or right side of the heart The bradycardia was of variable intensity and in a few cats entirely absent When blood pressure responses to $PGF_{2\alpha}$ were studied in different experiments before and after vagotomy or atropinization it appeared that the bradycardia if sufficient in magnitude was of quantitative importance in the second slow phase of the depression A tendency to tachyphylaxis however rendered such quantitative evaluations difficult

The cross perfusion experiments revealed that reflex inhibition of vasoconstrictor tone did not contribute to the hypotensive effects of i v injected $PGF_{2\alpha}$

Injection in the aorta or left ventricle caused only a small reduction in blood pressure. Thus the direct effect on the vessels seemed to be of minor importance. This conclusion is also supported by finding that i.a. injections of 1—10 μg $PGF_{2\alpha}$ into muscle, intestinal and renal vascular beds increased the blood flow only through the muscle.

An analysis of the blood pressure depressing action of $PGF_{2\alpha}$ in the cat thus showed that this effect was due to primarily a constriction of pulmonary blood vessels but also to some extent to the bradycardia.

In rabbits the effect of i.v. injected $PGF_{2\alpha}$ was also a marked, prolonged fall in blood pressure. The mechanism by which $PGF_{2\alpha}$ exerts its hypotensive effect seemed, however, to be different from that in the cat, as no changes in right ventricular pressure and heart rate were observed.

Bearing in mind the close chemical similarity of the lung factor (= $PGF_{2\alpha}$) and the prostaglandins isolated from sheep vesicular glands (BERGSTRÖM et al. 1962 a, b, c), it is of interest to recall some early observations by EULER (1939) with a purified extract of sheep glands. In perfused hindquarters of rabbits and cats this "prostaglandin" caused a clear vasodilation in the rabbit but had only weak vasodilator effects in the cat. These findings could provide an explanation for the different circulatory effects of $PGF_{2\alpha}$ in cats and rabbits found in the present investigation. EULER also consistently noted vasoconstrictor effects of "prostaglandin" in the isolated perfused lungs of both cats and rabbits. This would agree with the present in vivo data in cats but not in rabbits.

A comparison is also relevant between our results and those of BERGSTRÖM et al. (1959) with regard to the effects of the two crystalline prostaglandin factors PGE_1 and $PGF_{1\alpha}$. It appears that $PGF_{2\alpha}$ has the same range of biological activity as $PGF_{1\alpha}$, only being slightly more potent (Table I). An additional double bond at the place indicated in formula II thus seems to increase the smooth muscle stimulating activity.

LINN et al. 1961 described in a recent short communication the presence of smooth muscle stimulating activity, SRS, in an acidic lipid extract from normal swine lungs. A direct comparison between the preparation of LINN et al. and of PGF_2 will have to be made to establish whether the activity is due to the same compound.

Since $PGF_{2\alpha}$ has been isolated from normal lung tissue it is interesting that it exerts such profound effects on the pulmonary vascular system. However, too little is known of the biological actions, formation and release of this substance to justify any further speculation as to its physiological significance at this point.

This work is part of investigations supported by grants from Svenska Sällskapet för Medicinsk Forskning (E.Ä.), Public Health grant no. HE 06533-02 to professor B. Uvnäs (E.Ä.), Therese och Johan Anderssons minne (S.B.) and Gustaf och Tyra Svenssons minne (S.B.).

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Biotinidase Activity in Animal Tissues

By

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Abstract

Koivusalo, M. and J. Pispa. *Biotinidase activity in animal tissues.* Acta physiol. scand. 1963. *58*. 13—19. — Biotinidase activity of human serum and rat, guinea pig and rabbit serum, liver and intestine was determined using biocytin as substrate. The biotinidase activities of human serum and rat serum and tissues were of the same magnitude; in guinea pig serum the activity was about 10 per cent and in rabbit serum only 1 per cent of the activity in human and rat sera. Similar although less marked differences were found in the biotinidase activity of rat, guinea pig and rabbit tissues. The biotinidase activity of human and rat sera was inhibited by *p*-chloromercuribenzoate, arsenite and monoiodoacetate; it was slightly stimulated by glutathione. Ethylenediamine tetraacetate and cyanide had no effect. In zone electrophoresis the human serum biotinidase activity had slightly smaller mobility at pH 8.6 than albumin and moved as a single peak.

It is well established that biotin in cells and tissues is firmly bound to protein (György 1954). A simple biotin conjugate, biocytin, has been isolated in crystalline form from yeast extract (Wright *et al.* 1950, 1952) and identified as ε-N-biotinyl-L-lysine (Peck, Wolf and Folkers 1952), in which the carboxyl group of biotin is bound to the amino group of lysine through a peptide bond like linkage. In the known biotin enzymes, which catalyze carboxylation and transcarboxylation reactions, biotin is believed to be covalently bound to the protein, apparently to lysine residues (Lynen *et al.* 1959, 1961, Wakil and Gibson 1960, Kaziro, Leone and Ochoa 1960, Ochoa and Kaziro 1961).

Thoma and Peterson (1954) have found in hog liver and kidney an enzyme which releases free biotin from the bound forms after tryptic or peptic hydro-

lysis It also hydrolyzes biocytin and N biotinyl *p* aminobenzoate and was named biotinidase In human blood plasma WRIGHT, DRISCOLL and BOGER (1954) have described a similar enzyme which hydrolyzes biocytin and named it tentatively biocytinase A bacterial biotinidase has been partly purified and characterized from *Streptococcus faecalis* C10 using biocytin as substrate (KOIVUSALO *et al* 1963)

In this paper results from a survey of biotinidase activity in human serum and in the serum and tissues of some animal species are presented with some properties of the serum biotinidase In biotidinase activity assays biocytin has been used as substrate

Material and Methods

The biotinidase activity was assayed as follows The sample was incubated with 0 02 μmoles of biocytin and 40 μmoles of potassium phosphate buffer pH 7 0 in a final volume of 1 0 ml for 30 min in a waterbath at + 30 C The reaction was terminated by immersing the tube in a boiling waterbath for 2 min The coagulated protein was removed by centrifugation and the supernatant was assayed for free biotin

The free biotin was determined microbiologically using as the test organism *Lactobacillus arabinosus* 17 5 which does not respond to biocytin The assay was run essentially after the procedure of WRIGHT and SKEGGS (1944) Difco Bacto-Microinoculum Broth and Difco Biotin Assay Medium were used for the preparation of inoculum and as the assay medium respectively The tubes were incubated at + 30 C for 36—40 hours and the growth of the cells was measured in a Klett Summerson colorimeter using the red filter Standard assays were made with crystalline D biotin

One unit of biotinidase activity is defined as the amount of enzyme which liberates 1 mμmole of biotin per minute under the above conditions The specific activity is expressed as units per g of protein

The experimental animals were adult rats, rabbits and guinea pigs of both sexes from the laboratory stock The liver and a piece of intestine were removed immediately after killing the animal and cooled on ice The intestine was opened and gently flushed with ice-cold buffer The tissues were homogenized with 9 volumes of ice-cold 0 05 M potassium phosphate buffer pH 7 0 in a Potter Elvehjem type glass homogenizer The resulting preparations were immediately assayed for biotinidase activity as described above

The human serum samples were obtained from healthy donors and assayed immediately

The protein content of the tissue homogenates and sera was determined by the method of LOWRY *et al* (1951) using purified human albumin as a standard

The zone electrophoresis of serum was carried out using a rectangular Perspex through packed with polyvinyl chloride resin (Geon 426 G F Goodrich Co) moistened with barbital buffer pH 8 6 ionic strength 0 05 as described earlier (KOIVUSALO and PISPA 1958) The time of electrophoresis was 48 hours, a voltage of 400 V and a current of 10 mA were used per block After the run the resin block was cut in 0 6 cm fractions and each fraction was divided into two parts from which the enzyme activity and the protein content respectively were determined The protein content of the fractions was determined by the method of KUNKEL and TISELIUS (1951) after elution by shaking for 30 min with 3 ml of 0 1 N NaOH The enzyme activity was eluted by shaking for 30 min with 0 5 M potassium phosphate buffer pH 7 0

Table I Biotinidase activity of human and some animal sera

The activities are expressed as mμmoles of biotin liberated per minute and ml of serum in the standard assay conditions. The results are mean values of independent observations ± S. D (Range within brackets)

	No of observations	Biotinidase activity mμmoles/min/ml
Human serum	17	5.77 ± 0.90 (3.60 – 7.10)
Rat serum	19	5.63 ± 1.10 (3.57 – 8.60)
Guinea pig serum	12	0.583 ± 0.253 (0.167 – 1.190)
Rabbit serum	19	0.040 ± 0.010 (0.007 – 0.057)

Table II Biotinidase activity of liver and intestine homogenates of some animal species

The activities are expressed both as mμmoles of biotin liberated per minute and gram of fresh tissue and as mμmoles of biotin liberated per minute and gram of protein The standard conditions were used in the assays. The results are mean values of independent observations ± S D (Range within brackets)

Tissue	No of observations	Biotinidase activity	
		mμmoles/min/g of fresh tissue	mμmoles/min/g of protein
Rat liver	12	4.13 ± 0.83 (2.87 – 5.47)	20.7 ± 4.3 (13.0 – 27.7)
Guinea pig liver	10	1.85 ± 0.53 (1.17 – 2.77)	9.0 ± 1.7 (6.0 – 11.3)
Rabbit liver	6	1.10 ± 0.53 (0.63 – 2.07)	6.3 ± 2.3 (4.0 – 10.0)
Rat intestine	6	3.23 ± 0.77 (2.03 – 4.00)	27.3 ± 3.0 (22.7 – 31.7)
Guinea pig intestine	5	0.847 ± 0.133 (0.150 – 0.467)	8.0 ± 2.3 (5.7 – 11.0)
Rabbit intestine	6	0.250 ± 0.107 (0.150 – 0.467)	3.0 ± 0.7 (2.7 – 4.3)

Biocytin was kindly supplied by Dr Karl Folkers Merck Institute for Therapeutic Research Rahway New Jersey through the courtesy of Dr Severo Ochoa New York University The D-biotin used as standard was a crystalline preparation obtained from *Hoffmann La Roche Inc Nutley New Jersey The culture of Lactobacillus arabinosus* (ATCC *L. plantarum*, No 8014) was obtained from the American Type Culture Collection.

Results

A comparison of the biotinidase activities in human rat guinea pig and rabbit sera is presented in Table I The activity in the human sera was fairly constant and no sex linked differences could be observed The values obtained with rat sera were of the same magnitude as the values for human sera In guinea pig sera however the activity was only 10 per cent and in rabbit sera 1 per cent of that found in the human and rat sera

Table III Effect of some inhibitors and activators on the biotinidase activity of rat and human serum

The biotinidase activity is expressed as mμmoles of biotin liberated in 30 min in the usual assay conditions. The amount of serum was 0 01 ml The effect of additions on the growth of *L. arabinosus* was checked with the use of unincubated blanks.

	Additions	Biotinidase activity mμmoles/30 min	Per cent of controls
Rat serum	None	1 84	100
	Ethylenediamine tetraacetate 10^{-4} M	1 84	100
	Cyanide 10^{-3} M	1 95	106
	p-Chloromercuribenzoate 10^{-4} M	0 07	4
	p-Chloromercuribenzoate 10^{-5} M	1 63	89
	p-Chloromercuribenzoate 10^{-7} M	1 90	103
	Glutathione 6 10^{-3} M	2 43	13?
	Monoiodoacetate 10^{-2} M	1 16	63
	Arsenite 10^{-2} M	0 66	[illegible]6
Human serum	None	1 76	100
	Glutathione 10^{-3} M	2 06	117
	Glutathione 3 10^{-3} M	2 15	122
	p Chloromercuribenzoate 10^{-4} M	0 07	4
	p Chloromercuribenzoate 10^{-5} M	0 47	27
	p-Chloromercuribenzoate 10^{-7} M	1 69	96

A considerable biotinidase activity was also found in homogenates of rat liver and intestine as can be seen from Table II When homogenates of guinea pig and rabbit liver and intestine were incubated with biocytin some release of free biotin was regularly noted although the rate was markedly lower than with rat tissues The activities are given in Table II both per gram of fresh tissue and per gram of protein

A range of known enzyme inhibitors and activators has been tested for an effect on biotinidase activity in human and rat sera (Table III) The possible effect of these compounds on the response of *L. arabinosus* to biotin was also checked with the use of non incubated controls *p* Chloromercuribenzoate inhibits strongly monoiodoacetate and arsenite inhibit also but less markedly Ethylenediamine tetra acetate and cyanide had no effect The addition of reduced glutathione slightly stimulated the biotinidase activity

The apparent Michaelis constants (K_m) for biocytin has been determined with rat and human serum as sources of biotinidase The results obtained by plotting the reciprocal of the velocity of biotin liberation against the reciprocal of the biocytin concentration (LINEWEAVER and BURK 1934) gave K_m values (at 30) of 5 10^{-4} M and 2 10^{-4} M for rat and human serum respectively

Fig 1 Zone electrophoresis of human serum in a polyvinyl chloride block, with biotinidase activity and protein assays ——• biotinidase activity ---- protein Place of application is marked with arrow

Human serum was also subjected to zone electrophoresis in polyvinyl chloride resin (Geon 426) blocks and in addition to protein the biotinidase activity of the fractions was determined One typical electrophoresis curve is presented in Fig 1 The biotinidase activity moved as a single symmetrical peak and had slightly lower mobility at pH 8 6 than serum albumin

Discussion

The linkage between biotin and lysine in biocytin is very resistant to proteolytic and hydrolytic enzymes It is unaffected by e g pepsin trypsin papain takadiastase polidase and mylase (Wright et al 1950 1952) *Streptomyces griseus* protease which splits almost all peptide bonds in proteins and peptides (Nomoto Narahashi and Murakami 1960 a b) does not attack biocytin (Koslow and Lane 1962) Paik et al (1957) have described the presence of an ε lysine acylase in rat tissues which is distinct from the renal α acylase and hydrolyzes several ε acylated L-lysine derivatives but not biocytin ε lysine acylases have been described also in bacteria (Wada 1959 Chibata Ishikawa and Tosa 1962) but there is no information on their effects on biocytin The bacterial biotinidase from *S faecalis* (Koivusalo et al 1963) does not hydrolyze ε acetyl L-lysine and thus differs from the ε acylases

The present results corroborate the finding by WRIGHT *et al* (1954) that human blood contains an enzyme in considerable quantity that is active in hydrolyzing biocytin The biotinidase activity in rat serum was of the same magnitude as in human serum, but in guinea pig and rabbit sera there was only one tenth and one hundredth respectively of the activity in human serum Similar although less marked differences were found in the biotinidase activities of rat, rabbit and guinea pig tissues Liver and intestinal tissues were selected for the comparison because in a preliminary survey (KOIVUSALO *et al* 1963) they had shown high biotinidase activity in rat The activating effect of sulphydryl compounds on the serum biotinidase activity was not as marked in the present experiments as in those of WRIGHT *et al* (1954) The addition of reduced glutathione gave only a slight activation *p* chloromercuribenzoate iodoacetate and arsenite were, however inhibitory It seems very likely that the serum biotinidase activity is due to the presence of one single enzyme because the activity moves as a single symmetrical peak in zone electrophoresis Further characterization of the animal biotinidase cannot however, be made before purification and studies on the substrate specificity

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The Influence of Reserpine on 5-Hydroxytryptamine and Histamine Content of Rat Mast Cells and of Some Rat Tissues

By

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Abstract

MORAN N C and B WESTERHOLM *The influence of reserpine on 5-hydroxytryptamine and histamine content of rat mast cells and of some rat tissues* Acta physiol scand 1963 *58* 20—29 — Reserpine was shown to release neither 5 HT nor histamine from rat peritoneal mast cells *in vitro* *In vivo* reserpine caused a moderate reduction of the number of mast cells in peritoneal fluid A reduction of the cellular content of 5 HT was also observed Peritoneal mast cells obtained from reserpine treated rats responded normally to compound 48/80 Reserpinization of the rats caused reduction of 5 HT in brain but not in skin ileum duodenum pylorus or fundus Histamine was reduced to 35 per cent of control value in small intestine while no decrease was observed in vitro About 90 per cent of the rats showed ulcerations in the antral mucosa Pretreatment of the rats with compound 48/80 caused disappearance of all mast cells and of 5 HT and histamine from peritoneal fluid The results indicate that reserpine has no effect on mast cells *in vitro* and only negligible effects *in vivo* They also indicate that reserpine depletes the intestine of histamine which is of non mast cell origin

It is well known that reserpine releases 5 hydroxytryptamine (5 HT) from various 5 HT rich tissues such as rabbit intestine and brain (PLETSCHER SHORE and BRODIE 1955 BRODIE PLETSCHER and SHORE 1955) rabbit platelets (SHORE *et al* 1956) and rat serum spleen and brain (ERSPAMER 1956)

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In the rat mast cells contain 5 HT (BENDITT *et al* 1955) but there is no evidence that reserpine releases 5 HT from them Thus BHATTACHARYA and LEWIS (1956) found no release of 5 HT or histamine from rat hindquarters perfused with reserpine Nor did reserpine seem to disrupt the mast cells in the skin an observation which was confirmed by PARRATT and WEST (1957) and by FIORE-DONATI and MOLTKE (1960) It was also shown by CASS MARSHALL and RILEY (1958) that the 5 HT content of mast cells in mouse and rat bones did not change after reserpine treatment

Since none of these investigators studied the effect of reserpine on isolated mast cells we found it of value to investigate the influence of reserpine on mast cells in rat peritoneal fluid At the same time the influence of reserpine on the 5 HT and histamine content of some rat tissues was studied and the results compared with those obtained with compound 48/80

Methods

Male albino rats of the Wistar strain weighing 200—400 g were used The following types of experiments were performed

Incubation experiments

Incubation of rat peritoneal cells Rat peritoneal cells were obtained according to the method of MORAN UVNÄS and WESTERHOLM (1962) and the number of mast cells was estimated according to BRAY and VANARSDEL (1961) The cell suspensions contained 3—10 per cent mast cells

The cells were suspended in a solution containing NaCl 1.54×10^{-1} M KCl 2.7×10^{-3} M $CaCl_2$ 9×10^{-4} M 10 per cent Sorensen phosphate buffer (Na_2HPO_4 $2 H_2O$ 3×10^{-3} M and KH_2PO_4 3.5×10^{-3} M) pH 7.0 dextrose 5.6×10^{-3} M and serum albumin 1 mg per ml In some experiments cells from several animals were pooled The cell suspension was divided into 2 ml portions which were incubated at 37 C with reserpine or compound 48/80 After the incubation the samples were centrifuged at $400 \times g$ for 3 min and the 5 HT and histamine content of the supernatant and the cell residue was determined

Incubation of rat intestine The small intestine from rats starved for 24 hours was removed and cut into thin pieces which were carefully mixed The tissue was divided into 0.5 g portions which were incubated at 37 C in the medium mentioned above containing various concentrations of reserpine After incubation the 5 HT and histamine content of the medium was determined The tissue was homogenized with sand and extracted with 0.1 N HCl for 5-HT and histamine assay

Experiments with reserpinized rats

The rats were injected subcutaneously with reserpine 5 mg kg Control rats were injected with physiological saline After 24 hours the rats were anaesthetized with ether and killed by exsanguination The peritoneal cells were removed counted and incubated as in the experiments described above A piece of abdominal skin ileum, duodenum pylorus fundus and brain were taken out weighed and homogenized with sand in 0.1 N HCl for 5-HT and histamine extraction

Experiments with 48/80-treated rats

The rats were injected subcutaneously with compound 48/80 for 5 days beginning with 100 µg and increasing by 100 µg per day up to 500 µg Control rats were injected

Table I Influence of reserpine phosphate and compound 48/80 on 5 HT and histamine release from rat peritoneal cells Cells pooled from 5 animals Incubation time 5 hours Mean values of duplicate samples

Releasing agent	Per cent release		Amount of amines (μg) in cell fraction after incubation	
	5 HT	Histamine	5 HT	Histamine
Control	12	18	2 72	30.2
Reserpine phosphate				
2.5 $\mu g/ml$	12	19	2.38	2С 8
25 $\mu g/ml$	5	15	2.56	28.2
250 $\mu g/ml$	8	24	2.51	29 4
Compound 48/80				
2 5 $\mu g/ml$	60	66	0 91	9.2

with physiological saline On the sixth day the rats were killed and the cells and tissues were prepared in the same way as those of reserpinized rats

Determination of 5-HT and histamine

5-HT in cell suspensions and tissues was determined spectrophotofluorimetrically according to Bogdanski *et al* (1956)

Histamine was assayed on the atropinized (atropine sulfate 1 5 × 10^{-4}M) guinea pig ileum. Specificity was demonstrated by mepyramine block (5 × 10^{-7}M)

All 5-HT and histamine values are given as the base

Further details in the experimental procedures will be described in results

Materials

Reserpine phosphate was kindly supplied by Dr Albert J Plummer CIBA Pharmaceutical Products Inc Summit N J U S A It was used in an aqueous solution and no organic solvents were employed

Compound 48 80 was prepared according to the method described by Baltzly et al (1949)

Human serum albumin was generously supplied by Dr Hellstrom Kabi AB Strangnas Sweden

Other substances used were obtained from standard commercial sources

Results

Incubation of rat peritoneal cells with reserpine

Release of neither 5 HT nor histamine could be demonstrated from rat peritoneal cells when incubated with reserpine phosphate 2 5—250 $\mu g/ml$ up to 5 hours Nor was there any reduction of the amine content of the cells (Table I)

Table IIa Number of mast cells in rat peritoneal fluid and 5 HT and histamine content per million mast cells in control and reserpine treated (5mg/kg) rats Values given as means and standard errors Figures within brackets denote number of animals used

	No of mast cells	5 HT $\mu g/10^6$ mast cells	Histamine $\mu g/10^6$ mast cells
Control rats	678 000 ± 25 000 (49)	1.59 ± 0.19 (42)	39.3 ± 3.6 (47)
Reserpine treated rats	†455 000 ± 33 000 (42)	*1.04 ± 0.13 (37) <(0.36 — 3.33) (10)	30.9 ± 2.9 (42)

$p < 0.001$ compared with controls
* $p < 0.05$ compared with controls

Table IIb Number of mast cells in rat peritoneal fluid and 5 HT and histamine content per million mast cells in control starved and dehydrated and reserpinized (5 mg/kg) rats Values given as means and standard errors Figures within brackets denote number of animals used

	No of mast cells	5 HT $\mu g/10^6$ mast cells	Histamine $\mu g/10^6$ mast cells
Control rats	642 000 ± 36 000 (10)	1.07 ± 0.37 (7) <0.38 (3)	59.9 ± 11.6 (10)
Starved and dehydrated rats	697 000 ± 64 000 (10)	0.98 ± 0.08 (10)	69.7 ± 14.0 (10)
Reserpine treated rats	399 000 ± 71 000 (10)	0.24 (1) <(0.17 — 0.78) (9)	38.6 ± 6.2 (10)

$p < 0.01$ compared with controls

Table IIc Number of mast cells in rat peritoneal fluid and 5 HT and histamine content per million mast cells in control and 48/80 treated rats Values given as means and standard errors Figures within brackets denote number of animals used

	No of mast cells	5 HT $\mu g/10^6$ mast cells	Histamine $\mu g/10^6$ mast cells
Control rats	602 000 ± 46 000 (8)	1.08 ± 0.34 (8)	33.9 ± 4.7 (8)
Rats treated with compound 48/80	None found (8)	None detectable (8)	

Table III Influence of reserpine-treatment (5 mg/kg) on 5-HT and histamine content of rat skin, ... within brackets denote number of animals used

	Skin		Ileum		Duodenum
	5-HT µg/g	Histamine µg/g	5-HT µg/g	Histamine µg/g	5-HT µg/g
Control rats	1.13 ± 0.20 (14)	30.6 ± 4.5 (24)	2.9 ± 0.50 (28)	14.3 ± 1.6 (22)	3.28 ± 0.31 (31)
	± 0.5 (22)				
Reserpine treated rats	0.83 ± 0.10 (14)	31.3 ± 4.3 (24)	2.65 ± 0.5 (28)	5.0 ± 0.5 (26)	3.01 ± 0.32 (31)
	± 0.54 (2)				

[1] $p < 0.001$ compared with controls

Table IV Influence of pretreatment of rats with compound 48/80 on 5-HT and histamine content ... errors Figures within brackets denote number of animals used

	Skin		Ileum		Duodenum
	5-HT µg/g	Histamine µg/g	5-HT µg/g	Histamine µg/g	5-HT µg/g
Control rats	0.33 ± 0.04 (8)	**6.8 ± 1.9** (8)	1.0 ± 0.1 (8)	7.4 ± 1.4 (8)	1.16 ± 0.18 (8)
Rats treated with compound 48/80	0.29 ± 0.03 (8)	**2.3 ± 0.8** (8)	1.02 ± 0.10 (8)	11.3 ± 1.7 (8)	1.34 ± 0.17 (8)

[1] $p < 0.05$ compared with controls

Experiments with reserpinized rats

The reserpine treated rats showed the typical signs of reserpine action, e.g. lethargy, enophthalmus, ptosis, bloody diarrhea, weight loss and inflammation of eye lids and nose. About 90 per cent of the animals had ulcerations in the antral mucosa.

Pretreatment of the rats with reserpine caused reduction of the number of mast cells in the peritoneal fluid to 67 per cent (Table IIa). The 5-HT and histamine content per million mast cells was reduced to 65 and 79 per cent

ileum duodenum pylorus fundus and brain Values given as means and standard errors Figures

Duodenum	Pylorus		Fundus		Brain	
Histamine µg/g	5-HT µg/g	Histamine µg/g	5 HT µg/g	Histamine µg/g	5 HT µg/g	Histamine µg/g
10 7 ± 0 9 (31)	0 80 ± 0 19 (11)	11 6 ± 2 8 (11)	0 68 ± 0 09 (7)	5 4 ± 1 3 (5)	0.20 ± 0 04 (11)	0.31 ± 0 06 (7)
			< 0 41 (4)	< 0.32 (6)		< 0 09 (4)
3.5 ± 0 5 *(31)*	0 67 ± 0 17 *(11)*	9 7 ± 3 6 *(11)*	0 48 ± 0 08 (6)	4 6 ± 1 6 *(4)*	0 09 ± 0 01 (6)	0 46 ± 0 09 *(8)*
			< 0.26 (5)	< 0.23 (7)	< 0 06 (5)	< 0 03 (4)

* $p < 0\ 05$ compared with controls

of rat skin ileum duodenum pylorus fundus and antrum Values given as means and standard

Duodenum	Pylorus		Fundus		Brain	
Histamine µg/g	5 HT µg/g	Histamine µg/g	5-HT µg/g	Histamine µg/g	5-HT µg/g	Histamine µg/g
6 4 ± 0 5 (8)	1 24 ± 0.20 (8)	26.8 ± 3 8 (8)	1 02 ± 0.32 (8)	3 4 ± 1 0 (5)	0 20 ± 0 02 (8)	0 22 ± 0 08 (8)
				< 0.26 (3)		
8 6 ± 1 8 (8)	0 62 ± 0 15 (8)	25 9 ± 4 4 (8)	0 87 ± 0.28 (8)	3.9 ± 0 3 (4)	0 23 ± 0 04 (8)	0 35 ± 0 09 (8)
				< 0 27 (4)		

respectively as compared with the controls The reduction of histamine however was not significant

Since reserpinized rats lose weight and may be dehydrated a group of starved rats which had no water for 24 hours was compared with the controls (Table IIb) No difference in cell amount or amine content per million mast cells was observed between the two groups

The cell suspensions from the three groups were incubated with compound 48/80 1 µg/ml a dose which released about 70 per cent of the 5 HT and 80

per cent of the histamine in the cells in all three groups thus showing that the cells in the reserpinized group responded normally to compound 48/80

Pretreatment of the rats with reserpine caused no significant reduction of the 5 HT content of skin ileum duodenum pylorus or fundus (Table III) The brain content of 5 HT decreased to more than 45 per cent of control The histamine content of skin pylorus fundus and brain was unchanged while in duodenum it was reduced to 33 per cent and in ileum to 35 per cent of control

Incubation experiments with rat intestine

Since there was a significant reduction of the histamine content of the small intestine following pretreatment with reserpine the intestine from normal rats was incubated for 30 and 150 min with 1 and 100 μg reserpine phosphate per ml to evaluate release *in vitro* No significant 5 HT or histamine release was observed following this treatment Nor was there any reduction of the amine content of the intestine

Experiments with 48/80 treated rats

Pretreatment of the rats with compound 48/80 caused disappearance of all the mast cells in the peritoneal fluid Other cells such as leucocytes lymphocytes and red cells occurred in a larger amount than in the controls Neither 5 HT nor histamine could be demonstrated in the peritoneal fluid (Table IIc)

The 5 HT content was unchanged in skin ileum duodenum fundus and brain while in pylorus a reduction to 50 per cent was demonstrated (Table IV) Histamine was reduced to 34 per cent in skin No reduction was seen in other tissues

Discussion

The inability of reserpine to induce release of 5 HT and histamine from rat peritoneal mast cells *in vitro* supports the observation of BHATTACHARYA and LEWIS (1956) that administration of reserpine to the perfused hindquarters of the rat releases neither of the amines Since in both of these preparations compound 48/80 evokes a release of both substances the conclusion seems apparent that reserpine lacks a specific amine releasing action on mast cells In further agreement are the observations of BHATTACHARYA and LEWIS that pretreatment with reserpine causes no degranulation of mast cells in the subcutaneous tissue of mesentery of rats Similar observations on the lack of effect of reserpine on skin mast cells were subsequently made by PARRATT and WEST (1957) and FIORE DONATI and MOLTKE (1960)

Whether reserpine influences mast cells *in vivo* is uncertain Our results show that pretreatment of rats with reserpine reduces the number of cells in peritoneal fluid as well as the cellular content of 5 HT but does not alter the ability

of compound 48/80 to induce release of the amines These effects may be secondary to changes in the state of the animals produced by reserpine The failure of deprivation of water and food to alter the mast cells argues against dehydration as a cause However the effects of large doses of reserpine are probably more drastic than fasting and witholding water

In contrast to the effect of reserpine on mast cells is that on platelets Reserpine induces release of 5 HT from platelets *in vitro* (CARLSSON SHORE and BRODIE 1957) but not of histamine (BURKHALTER, COHN and SHORE 1960) Compound 48/80 on the other hand, causes no release of 5-HT from platelets *in vitro* (CARLSSON *et al* 1957) The release of 5-HT from platelets *in vitro* is a slow process of zero order kinetics with no accompanying disruption of the cells (CARLSSON *et al* 1957) in contrast to the explosive 48/80-induced release of both 5-HT and histamine from mast cells We know of no data on 48/80-induced histamine release from platelets

According to the work of BHATTACHARYA and LEWIS (1956) pretreatment of a rat with reserpine alters the response to 48/80 in the perfused hindquarters such that histamine is still released but the amount of 5-HT released is greatly reduced compared with normal rats This action may be related to our observation that reserpine administration to living rats reduces the number of mast cells in the peritoneal fluid and the cellular content of 5 HT However it does not cast any light on the difference in action between *in vivo* and *in vitro* conditions

These results point to the conclusion that whatever effects reserpine has on mast cells these effects are of small magnitude in contrast to the effect of compound 48/80 on mast cells

Our finding of a selective reduction of histamine in the doudenum and ileum (and possibly in the pyloric part of the stomach) following treatment of rats with high doses of reserpine is of interest in relationship to the genesis of the peptic ulcers produced by reserpine and to the findings of MOTA *et al* (1956) Regarding the latter MOTA *et al* treated rats with daily injections of 48/80 and then measured the content of histamine and the relative number of mast cells in various tissues They found the greatest depletion of histamine in those tissues with the highest mast cell populations such as skin tongue and esophagus whereas little or no depletion of histamine occurred in tissues with few or no mast cells such as gastric fundus duodenum ileum and rectum MOTA *et al* suggest that the inability of 48/80 to release histamine from tissues poor in mast cells is due to this histamine being of non mast cell origin The selective depletion of histamine by reserpine in these tissues which are refractory to 48/80 gives added support to this concept

The ulceration of the antral region of the stomach of rats during treatment with reserpine has been observed by others (HAVERBACK and BOGDANSKI 1957 KEYRILAINEN KALLIOMAKI and GRÖNROOS 1957 BLACKMAN CAMPION and

FASTIER 1958) The relationship of this to the depletion of histamine found in the duodenum and ileum is however, not clear

The absence of an effect of reserpine on intestinal 5 HT is in agreement with the finding of ERSPAMER (1956) that only a small part of intestinal 5 HT disappeared following treatment of rats with reserpine SANYAL and WEST (1958) on the other hand reported depletion of rat intestine 5 HT by pre treatment with reserpine These authors however, give no values for the 5 HT content of the tissue Other workers (PLETSCHER *et al* 1955, WAALKES COBURN and TERRY 1959) demonstrated a reduction of intestinal 5 HT in rabbits using doses of reserpine of the same order as we used It is possible that the rabbit is more sensitive to reserpine than the rat

It is concluded that reserpine and compound 48/80 cause the release of 5-HT and histamine from cellular stores but under different conditions and from different cells Compound 48/80 acts rapidly both *in vivo* and *in vitro* on mast cells but presumably not on other cells causing release of both 5 HT and histamine and disruption of the cell Reserpine has no effect on mast cells *in vitro* and slight if any effect on these cells *in vivo* However reserpine effects a slow release of 5-HT from platelets and a slow depletion of histamine from the intestine and of 5-HT from such tissues as brain and skin The characteristics of the amine release induced by these two agents have little in common to suggest a similar mechanism of action

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Effects of ACTH on Erythropoiesis in the Rabbit

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Abstract

Halvorsen, S. *Effects of ACTH on erythropoiesis in the rabbit.* Acta physiol. scand. 1963. *58*. 30—39. — Hypothalamic stimulation increases erythropoiesis in intact rabbits (Seip *et al.* 1961). In order to compare the effect of hypothalamic stimulation on erythropoiesis with the effect of ACTH, daily ACTH injections have been given to intact rabbits and the changes in erythropoiesis have been followed with the same parameters as in the previous study. ACTH injections for 2 and 4 weeks increased the red cell mass 11.2 and 27.7 per cent, and the red cell mass per kg body weight 6.3 and 23.3 per cent, respectively. *The reticulocytes increased slightly but significantly.* These findings confirm previous observations in other species that ACTH stimulates erythropoiesis in intact animals. The hematological changes seen in the ACTH-injected rabbits did not correspond quantitatively to the changes seen following hypothalamic stimulation. It is concluded that the previously reported effect of hypothalamic stimulation on erythropoiesis is not due to increased ACTH production alone.

In a previous paper Seip *et al.* (1961) have presented data showing that electrical stimulation of the hypothalamic region stimulates erythropoiesis in the rabbit. Based on data obtained in the same animals Halvorsen (1961) suggested that the effect of hypothalamic stimulation on erythropoiesis was mediated through humoral agents and not through a direct nervous influence on the bone marrow. The nature of the humoral agents cannot be stated with certainty, but as pointed out previously (Halvorsen 1961) they are probably specific erythropoiesis stimulating factors (ESF). The exact mechanism by which an increased ESF production may be elicited by electrical stimulation of the hypothalamic region is not known. There are two main possibilities: either a direct influence via the nervous system to the erythropoietin producing

organs or an indirect effect via ACTH or other hormones of the pituitary gland

The question then arises whether the increase in reticulocytes red cell mass (RCM) and ESF by hypothalamic stimulation can be explained by an increase in ACTH production alone If this is the case one would expect the same hematological changes as reported by SEIP *et al* (1961) and HALVORSEN (1961) by daily ACTH injections for a similar period of time (2 weeks) It is the purpose of the present experiments to determine the effect of daily injections of ACTH on erythropoiesis in rabbits

It has been documented that stimulation of the hypothalamus increases the ACTH production (MASON 1958) A similar but less marked increase has been found following stimulation of certain areas of the cerebral cortex and the amygdala (SETEKLEIV SKAUG and KAADA 1961) Hypothalamic lesions in the median eminence and in the postoptic area abolish the increase in plasma ACTH and 17 hydroxycorticosteroid secretion resulting from surgical trauma (HUME 1958)

The ACTH and the adrenal corticosteroid production has probably been increased in the rabbits receiving electrical stimulation of the hypothalamus (SEIP *et al* 1961) Judged on indirect evidence such as white blood cell and differential counts no such increase could be demonstrated However as these indirect evidences are unreliable and have only limited value one must assume that the stimulated rabbits were influenced by increased amounts of ACTH

Previous investigations have demonstrated that ACTH in large doses increases erythropoiesis in intact mice and rats WHITE and DOUGHERTY (1945) observed an increase in hemoglobin erythrocytes and specific gravity of whole blood following ACTH administration to mice for 15 days On the other hand GARCIA *et al* (1951) found no obvious increase in red cell volume after 34 days of ACTH administration but a significant increase after 116 days in normal rats Although it has been postulated that the ACTH preparations have contained a separate erythropoiesis stimulating hormone (CONTOPOULOS *et al* 1953) studies in the same laboratories have recently shown that the effects of ACTH are abolished in adrenalectomized animals (EVANS, ROSENBERG and SIMPSON 1961) Thus the effect of ACTH is most likely mediated through the adrenal corticosteroids Cortisone and hydrocortisone increase erythropoiesis in intact rats (FISHER 1958) and normalize the anemia in hypophysectomized rats (EVANS *et al* 1961) The effect of the corticosteroids parallel the increase in the metabolic rate of the animals (EVANS *et al* 1961)

In contrast to EVANS *et al* (1961) FRUHMAN and GORDON (1956) and MEINEKE and CRAFTS (1957) did not find that adrenal steroids completely corrected the post hypophysectomy anemia The discrepancy between these results may be due to differences in preparations and dosage

Some authors (EVANS *et al* 1961) believe that the influence of ACTH and adrenal corticosteroids on erythropoiesis is of secondary nature due to their

calorigenic effect. OSNES (1960), on the other hand, maintains that ACTH stimulates the production of an erythropoietic kidney factor and this theory has been supported by the findings of SHIRAKURA (1961).

The growth hormone (FRUHMAN and GORDON 1956), thyroxin (EVANS *et al* 1961) and testosterone (KENNEDY and GILBERTSEN 1957, REISSMANN *et al* 1961) similarly have a stimulating effect on erythropoiesis, but the relative importance of the various hormones remains unknown. The problem of the endocrine control of erythropoiesis has been reviewed by CRAFTS and MEINEKE (1957), GORDON (1959) and LINMAN and BETHELL (1960) and will not be further discussed in this connection.

Material and Methods

Eleven male rabbits have been injected daily with ACTH. Seven of the rabbits were injected 14 days and four 28 days. In two of the latter animals RCM was also determined after 14 days of ACTH injections, and the observations on the first 2 week period are presented together with the results on the seven rabbits injected 14 days, making the number in this group nine. Two ACTH preparations have been used. Jaton prolongatum[1] has been given in doses of 4 and 10 I. U./kg body weight/day and Acthar gel[2] in doses of 4 I. U./kg body weight/day.

Eight male rabbits have served as controls. Except for the ACTH injections the diet, the animals' daily activities and the experiments have been the same. Control rabbits no. 1 and 2 were followed for two weeks, no. 3 and 4 for four weeks and in no. 5, 6, 7 and 8 the RCM was determined after two and four weeks. Thus the control group consists of 10 observations on the hematological changes during a period of two weeks and 6 observations on the changes during four weeks.

In both groups RCM, hemoglobin, hematocrit, erythrocytes, leucocytes, differential counts and reticulocytes were determined at the outset of each observation period, and the same investigations were repeated at the end of each period. The reticulocytes were counted two to three times weekly during the experiments. Blood for erythropoietin determinations was withdrawn three to five hours following the last ACTH injection in 5 rabbits.

Hemoglobin, erythrocytes, leucocytes and differential counts were performed by the same routine methods as in the previous investigations (SEIP *et al.* 1961). Hematocrit was determined using capillary tubes run in a Ljungberg microhematocrit centrifuge. The hematocrit had to be determined for the RCM analysis, and the mean of the values expresses the hematocrit at each time.

Red cell mass (RCM) was determined by the Cr^{51} method, as described by SEIP *et al.* (1961), with some modifications. In 10 of the rabbits two blood samples were taken before the injection of the tagged erythrocytes if RCM had previously been determined in the same animal, and three samples after the injection. With this modification another 4 double determinations were done. The greatest difference between the two values was 3.6 per cent of the initial value.

The reticulocytes were counted using the brilliant cresyl blue method (SEIP 1953). Five thousand erythrocytes were counted. The erythropoietin determinations were performed with starved mice and intact mice as described by HALVORSEN (1961).

[1] Jaton prolongatum, Apotekernes Laboratorium, Oslo, Norway.
[2] Acthar gel, The Armour Laboratories, London, England.

Table I RCM RCM/kg body weight and hematocrit before and after a 2 week period of daily ACTH injections 4 I U /kg body weight

ACTH treated group. Two weeks.

Rabbit no	RCM (ml)		RCM/kg (ml)		Hematocrit	
	Before ACTH	After ACTH	Before ACTH	After ACTH	Before ACTH	After ACTH
1	35.8	38.4	16.4	16.6	34.0	33.5
2	52.7	51.4	20.3	20.2	43.5	47.0
4	48.2	58.2	18.1	19.3	36.0	38.5
5	42.7	46.6	17.3	18.7	37.0	38.5
6	46.7	56.7	15.0	16.8	31.0	31.0
7	65.9	65.4	20.1	20.4	37.0	37.3
8	44.0	5[illegible].3	14.7	17.2	31.5	33.0
12 a	63.8	75.2	17.4	19.8	35.9	38.6
13 a	61.8	67.2	17.3	17.7	35.3	37.3

Table II RCM RCM/kg body weight and hematocrit determinations in control rabbits performed with an interval of two weeks

Control group. Two weeks.

Rabbit no.	RCM (ml)		RCM/kg (ml)		Hematocrit	
	Before	After	Before	After	Before	After
1	64.5	56.5	14.9	14.1	34.8	33.7
2	80.2	79.9	14.8	14.5	40.0	36.2
5 a	43.5	49.0	20.9	20.7	43.8	36.9
5 b	49.0	55.9	20.7	19.9	36.9	35.2
6 a	63.2	50.4	18.8	14.0	41.8	37.6
6 b	50.4	52.0	14.0	13.7	37.6	36.3
7 a	40.3	49.4	15.8	17.4	33.7	38.0
7 b	49.4	51.9	17.4	17.3	38.0	37.1
8 a	49.8	59.0	15.1	16.6	33.3	38.5
8 b	59.0	63.6	16.6	16.7	38.5	40.3

Results

In the beginning of the experiments the mean RCM and RCM/kg body weight in the ACTH treated group (2 weeks) were 51.3 ml and 17.4 ml respectively and in the controls 55.1 ml and 16.9 ml The mean reticulocyte values in the ACTH treated group were 2.82 per cent and in the controls 2.76 per cent The two groups are thus well comparable with regard to the main parameters at the start of the studies

Table I shows the RCM and hematocrit values in the rabbits before and after a 2 week period of daily ACTH injections The RCM kg body weight is

Table III RCM RCM/kg body weight and hematocrit before and after a 4 week period with daily ACTH injections

ACTH treated group Four weeks.

Rabbit no	RCM (ml)		RCM/kg (ml)		Hematocrit	
	Before ACTH	After ACTH	Before ACTH	After ACTH	Before ACTH	After ACTH
10	44.5	60 2	16 9	22.2	31 5	33 0
11	81 1	107 0	21 9	25.5	38 0	38.5
12	63 8	84 1	17 4	22 4	35 9	39 4
13	61 8	72 8	17 3	20.2	35 3	37.3

Table IV RCM RCM/kg body weight and hematocrit determinations in control rabbits performed with an interval of four weeks

Control group Four weeks.

Rabbit no	RCM (ml)		RCM/kg (ml)		Hematocrit	
	Before	After	Before	After	Before	After
3	56.2	63 8	18.6	17 4	35 0	35 9
4	45 6	61 8	15 2	17.3	32 3	35 3
5	43 5	55 9	20 9	19 9	43 8	35.2
6	63.2	57 0	18 8	13 7	41 8	36 3
7	40 3	51 9	15 8	17 3	33 7	37 1
8	49 8	63 6	15 1	16 7	33.3	40 3

calculated and tabulated Table II shows the same studies in the intact, untreated rabbits performed with 2 weeks interval Table V summarizes the results of the investigations showing the mean of the differences between the post and pre treatment values and the differences between the mean changes in the ACTH and the control groups

Treatment with ACTH for two weeks increased total red cell mass 5 5 ml compared to an increase of 1 8 ml in the controls The difference between the two groups is insignificant The RCM/kg body weight increased 1 1 ml in the ACTH treated group and decreased 0 4 ml in the controls The difference between the two groups is significant with a p value approximating 0 05 The hematocrit hemoglobin and erythrocytes did not show significant changes

Table III gives the values of RCM RCM/kg body weight and hematocrit in four rabbits receiving ACTH for 4 weeks and Table IV shows the same studies in six control rabbits performed with 4 weeks interval The results of these investigations are summarized in Table V

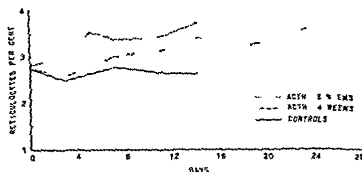


Fig 1 Mean of reticulocyte percentages at different times for
a) 9 rabbits receiving ACTH for 2 weeks
b) 4 rabbits receiving ACTH for 4 weeks
c) 8 control rabbits followed for 2 weeks

After 4 weeks of ACTH injections there was a marked increase in total red cell mass and RCM/kg body weight 16.5 ml and 4.2 ml respectively In the controls the corresponding figures were 8.4 ml and —0.4 ml The increase in RCM/kg body weight was significant compared with the controls (p approximating 0.05) The hematocrit hemoglobin and erythrocytes increased 9.4 8.3 and 9.3 per cent of the initial values in the ACTH treated group but the

Table 5 Mean of the differences between the post and pre treatment values (± S E) The change in per cent of the initial value is expressed The differences of the means between the ACTH treated and the control groups are tabulated in the third column

Parameter	2 weeks			4 weeks		
	ACTH (9)	Control (10)	ACTH control	ACTH (4)	Control (6)	ACTH control
RCM ml	5.5 ± 1.5 11.2	1.8 ± 2.2 3.3	3.7	17.0 ± 2.6 27.7	8.4 ± 4.1 16.8	8.6
RCM/kg ml	1.1 ± 0.3 6.3	−0.4 ± 0.5 −2.3	1.5	4.2 ± 0.5 23.3	−0.4 ± 1.1 −2.3	4.6
Hematocrit %	0.9 ± 0.5 2.?	−0.9 ± 1.1 −2.4	1.8	3.1 ± 1.3 9.4	0.0 ± 2.5 0.0	3.1
Hemoglobin g	−0.17 ± 0.3 −1.3	0.01 ± 0.3 0.8	−0.16	0.9 ± 0.2 8.3	0.0 ± 0.4 0.0	0.9
Erythrocytes, mill.	−0.17 ± 0.2 −3.2	0.19 ± 0.3 3.7		0.47 ± 0.3 9.3	0.11 ± 0.4 ?8	0.33

$p \leqq 0.01$ $0.05 > p > 0.01$ 0.1

Table VI Reticulocyte responses in intact mice following one injection of plasma from ACTH treated rabbits on day 0

Donor	No of mice	Reticulocytes			Mean diff ± S E (4th day—0 day)
		0	4	10 day	
ACTH 5	6	2 88	3 73	2 47	+0 85 ± 0 46
ACTH 10	7	3 60	4 83	—	+1 23 ± 0 72
ACTH 11	5	4 21	3 17	1 96	−1 04 ± 0 45

Table VII Fe^{59} per cent in the erythrocytes of starved mice injected with plasma (0 4 ml × 2) from ACTH treated rabbits and control rabbits

Donor	No of mice	Fe uptake Mean ± S E	Diff of the means
ACTH 11	4	26 4 ± 4 1	− 3 9
Control	4	30 3 ± 5 7	
ACTH 12	3	28 7 ± 9 7	+ 3 2
Control	3	25 5 ± 2 1	
ACTH 13	3	33 7 ± 1 8	+ 4 3
Control	3	29 4 ± 2 7	

differences between the values in this group and in the control group are in significant

Fig 1 shows mean of the reticulocyte values For each rabbit receiving ACTH for 2 weeks and for each control rabbit a straight line was fitted to the reticulocyte percentages The number of observations per rabbit varied from four to seven The regression lines for the 9 ACTH rabbits did not vary significantly among themselves and the same held true for the 8 regression lines for the controls A common regression coefficient for the ACTH rabbits was estimated as 0 55 per cent per day and for the controls as — 0 88 per cent per day These *coefficients differ significantly* ($0\ 005 > P > 0\ 001$)

Table VI gives the results of the erythropoietin determination using intact mice as recipients and in Table VII are reported the results with starved mice In the intact mice assays plasma from ACTH injected rabbits no 5 and 10 increased the reticulocytes slightly but insignificantly The reticulocyte values showed a mild decrease in the group of mice receiving plasma from ACTH injected rabbit no 11 In the starved mice assays plasma from ACTH injected rabbit no 11 did not cause any increase in Fe^{59} erythrocyte uptake while plasma from no 12 and 13 increased the iron uptake slightly but insignificantly

Comments

The present study confirms that ACTH may have a stimulating effect on erythropoiesis It has been shown that daily ACTH injections stimulate erythropoiesis in intact rabbits as previously shown in mice (WHITE and DOUGHERTY 1945) and rats (GARCIA *et al* 1951) The increase in erythrocyte formation is however slight in a 2 week period the mean increase in RCM kg body weight was 6 3 per cent In the controls the RCM/kg body weight decreased 2 3 per cent Following a 4 week period of ACTH injections a more marked increase in RCM/kg was found i e 4 2 ml (23 3 per cent) compared with the decrease of 0 4 ml (2 3 per cent) in the controls

As noted in other species (GARCIA *et al* 1951) the effect of ACTH on erythropoiesis is slow This is clearly demonstrated in the present study by the mild and gradual increase in reticulocytes in the ACTH injected animals This type of reticulocyte response was to be expected when the causative mechanism is metabolic changes with slowly increasing oxygen need The reticulocyte responses in the ACTH injected animals differ from the responses seen following hypothalamic stimulation (SEIP *et al* 1961) In some of the latter animals the reticulocytes showed abrupt rises in contrast to the slow rise in the rabbits receiving ACTH

Mean increase in total RCM in the group showing a positive response to hypothalamic stimulation was 9 1 ml (16 7 per cent) compared with 5 5 ml (11 2 per cent) following ACTH injections As the positive response group reported in the previous study was a selected group a statistical analysis would not be justifiable The ACTH treated animals gained more weight than did the electrically stimulated rabbits Therefore a more important comparison is between the increases in RCM/kg body weight In the positive response group of the previous study the mean increase in RCM/kg was 3 2 ml (18 4 per cent) compared with 1 1 ml (6 3 per cent) in the present study This further indicates that the ACTH injections during a 2 week period did not bring out the same quantitative changes as did hypothalamic stimulation In the negative response group the RCM/kg decreased 1 1 ml and in the doubtful response group the RCM/kg increased 1 4 ml

In previous investigations on the effects of ACTH and corticosteroids on erythropoiesis the size of the doses have varied In the study of EVANS *et al* (1961) a relationship between the doses used and the increase in red cell volume can be found Increasing doses of hydrocortisone did not however cause a parallel elevation of red cell volume when the doses were increased beyond certain levels In the present study the doses used per 100 g body weight can be compared with the doses used by EVANS *et al* (1961) to hypophysectomized rats Although intact rabbits have been used in the present study it is not likely that higher doses would have increased the red cell mass significantly The two rabbits receiving 10 I U ACTH per kg body weig 2 weeks showed a rise in RCM of 12 0 and 8 3 per cent of the initial val -

The hematocrit, hemoglobin and erythrocytes showed less distinct changes both in the ACTH treated animals and following hypothalamic stimulation After 2 weeks of ACTH injections the hematocrit values increased 2 7 per cent and the hemoglobin and the erythrocytes decreased 1 3 and 3 2 per cent The rise in RCM without a rise in hemoglobin and red blood cells may be due to simultaneous increases in the plasma volume, masking the increase in red cell volume In the rabbits receiving ACTH through 4 weeks an increse in hemoglobin and erythrocytes was noted but the data in this group are few

Plasma from the ACTH injected rabbits did not significantly increase Fe^{59} per cent uptake in the erythrocytes of starved mice or the reticulocytes of intact mice Because our present methods for erythropoietin determinations are relatively crude and insensitive, the slight increases noted in some of the assays are hardly significant, although it is possible that the effect of ACTH injections is mediated through small increases in erythropoietin production

When one compares the RCM RCM/kg body weight, reticulocytes and plasma erythropoietin levels in the ACTH treated group and in the positive response group of our previous study (Seip *et al* 1961), it is evident that the ACTH injections did not have the same quantitative effects as hypothalamic stimulation From this can be concluded that the hematological changes following hypothalamic stimulation are not due to increased ACTH production alone Hypothalamic stimulation may however also increase other pituitary hormones in addition to ACTH (Harris 1958) and the possibility of a combined effect of several pituitary hormones is open to discussion Another possibility, is as mentioned previously the existence of a hypothalamic area controlling erythropoietin production *via* the nervous system to the erythropoietin producing organ(s)

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Material

The animals were young gulls (*Larus argentatus* and *L. fuscus*) with a weight of about 1 kg and 9 to 11 weeks old ducks (*Anas platyrhynchus*) weighing about 3 kg

Methods

Anesthesia 1 ml 6 % Nembutal (Abbott) was injected into the median foot vein. To obtain even anesthesia 0 2 ml were added when required The amount of Nembutal used to produce anesthesia was the same for gulls and ducks in spite of the greater body weight of the latter The animals were tracheotomized and given artificial respiration

Blood pressure recording A strain gauge type transducer (Statham P 23) was connected by polyethylene tubing with a wing artery (*arteria brachialis*) The fluid space of the transducer and the tubing contained heparinized saline The transducer impulses were recorded by a Sanborn multichannel oscillograph

Polarography The oxygen electrode (cathode) consisted of a 0 1 mm platinum wire which was inserted into the tissue to be studied (KROG and JOHANSEN 1960) A silver silver chloride reference electrode in contact with the pectoral muscle served as anode The electrodes were connected with the Sanborn oscillograph *via* a special made polarograph AC—DC amplifier The electrode function was tested by asphyxiation of the animals through clamping of the tracheal cannula (Fig 1) Electrodes with slow response were replaced

Nerve stimulation Stimulation of the secretory parasympathetic nerve was made in gulls The peripheral stump of the severed nerve was used for stimulation

The cervical sympathetic trunk was reached at the level of the 6th vertebra The nerve is easily exposed in gulls but is less accessible in the duck due to the surrounding bony structure The cranial end of the cut nerve was stimulated Characteristics of the stimulation Frequency 20 sec, duration 1 msec intensity 1—6 volts

Pharmacological agents Small amounts of saline containing dissolved substances were injected intravenously *via* a catheter in a wing vein (*v brachialis*) or intra arterially In the latter case a T-shaped polyethylene cannula was placed in the carotid artery of one side In a few cases the injections were made *via* a side branch of the carotid artery The following substances were used acethylcholine chloride l adrenaline chloride histamine phosphate isoproterenol chloride metacholine chloride (Mecholyl) l noradrenaline chloride serotin creatinine sulfate Doses are expressed as μg of the salts

We acknowledge with thanks the gift of a sample of serotonin from the Upjohn Co Kalamazoo Mich and isoproterenol from Sterling Winthrop Research Inst Rensselaer N Y

Results

In anesthetized ducks or gulls not otherwise interfered with the blood pressure is about 150 mm Hg but considerable individual variations and slow spontaneous changes of blood pressure may occur

Changes in available oxygen (= oxygen tension) in the salt gland occur during asphyxia stimulation of the glandular nerves and under the influence of pharmacological agents The oxygen usually varies parallel to the blood pressure but under the influence of certain kinds of stimuli the variations in oxygen do not follow the blood pressure probably due to local vascular responses in the gland different from that of the rest of the body (Fig 2—5) See further the discussion p 46

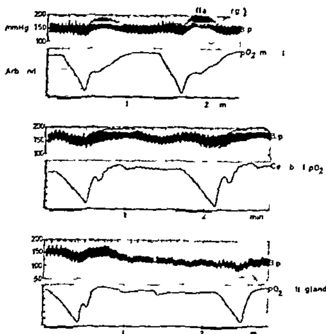


Fig 1 Control of the function of oxygen electrodes by asphyxiation At the signals (lowest line) the tracheal cannula was clamped The rapid decline of available oxygen indicates appropriate function of electrodes placed in a leg muscle the cerebral cortex and the salt gland Animal gull (*Larus argentatus*)

Asphyxia Asphyxiation causes a rapid fall in available oxygen in the salt gland and usually a slight increase of the blood pressure Analogous results are obtained with the oxygen electrode in the cerebral cortex or in a skeletal muscle (Fig 1) After establishment of normal air passage the available oxygen rises to the pre asphyxial level Identical results are seen in ducks and gulls

Stimulation of the cervical sympathetic trunk

In gulls and ducks sympathetic stimulation produces a very rapid decline of available oxygen in the salt gland and a small increase of blood pressure (Fig 2 1, 2) After ended stimulation the oxygen tension quickly goes up to the previous level *The results indicate that the cervical sympathetic system* carries vasoconstrictor fibres to the blood vessels of the salt gland The constrictor innervation is unilateral as shown from the following experiment on a duck If the oxygen tension is recorded from the salt gland and the contralateral cervical sympathetic trunk is stimulated only an extremely slight increase in oxygen, probable due to an increase in blood pressure is seen

Stimulation of the parasympathetic secretory nerve

The branch of the facial nerve carrying secretory fibres to the salt gland according to our findings also contains sympathetic elements Stimulation of

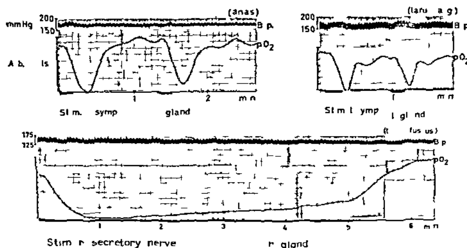


Fig 2 Effects of nerve stimulation on the available oxygen in the salt gland and the blood pressure in the subclavian artery 1) and 2) Sympathetic stimulation in periods of about 10 seconds 3) Parasympathetic stimulation in 5 minutes

this nerve causes contraction of cutaneous muscles in front of the eye These muscles are sympathetically innervated (LANGLEY 1903) Whether the nerve gives off sympathetic fibres to the gland is not known

Previous studies have shown that stimulation of the secretory nerve produces a flow of secreted fluid in the lateral duct of the salt gland (FANGE *et al* 1958) There is reason to think that the glandular lobes which connect with the median duct are innervated in the same way by the same nerve as those empty ing by the lateral duct Due to difficulties in cannulating the median duct this has not been verified so far In the present investigation no attempt was made to place the oxygen electrode exactly into a preselected part of the salt gland

Results from stimulation of the secretory nerve are seen in Fig 2 3 The available oxygen decreases rapidly during the first minute but at a slower rate than during sympathetic stimulation If stimulation is kept up for several minutes a very slow linear rise of oxygen takes place (Fig 2 3) A copious secretion of fluid is seen After ended stimulation the oxygen reaches a higher level than before the stimulation

Interpretation of the results The initial decrease of available oxygen may be due to vasoconstriction or to an increased metabolic consumption of oxygen during secretion or to both factors in combination The fact that the decrease of oxygen takes place at a much slower rate and lasts longer than the decrease of oxygen after sympathetic stimulation speaks against a nervous vasoconstric tion The slow rise of available oxygen after the first minute of stimulation and the continued rise after ended stimulation to a level above the prestimulatory one indicates a vasodilatation

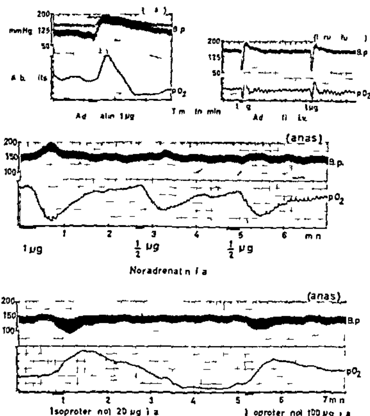


Fig 3 Effects of adrenaline noradrenaline and isoproterenol on the available oxygen in the salt gland and the blood pressure

Effects of drugs

Adrenaline and noradrenaline 1 µg i v of adrenaline or noradrenaline produces a moderate rise of blood pressure in the duck (Fig 3 1) and a rise preceded by an initial sharp decrease in the gull (Fig 3 2) The changes of oxygen closely follow the blood pressure I v doses of 10 µg or more of the catecholamines elevate the blood pressure but cause a decrease of the oxygen available in the salt gland The decrease of oxygen is presumably due to a local vasoconstriction

The local constrictory influence of catecholamines is more clearly seen after intra arterial administration Thus in the duck 0 5 or 1 µg of noradrenaline i a causes a large fall of oxygen concomitant to a rise in the blood pressure (Fig 3 3) Our data are insufficient but we have the impression that noradrenaline is slightly more active than adrenaline as a vasoconstrictory substance

Isoproterenol (N isopropyl noradrenaline) 1 to 10 µg isoproterenol i a cause a rise of oxygen but does not influence the blood pressure Higher doses give a

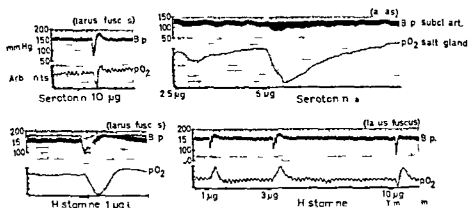


Fig 4 Effects of serotonin and histamine on the available oxygen and the blood pressure

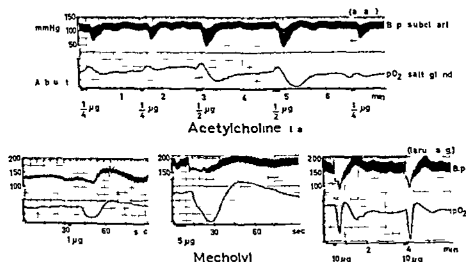


Fig 5 Effects of acetylcholine and mecholyl on the available oxygen, and the blood pressure

rise of oxygen and fall of blood pressure (Fig 3 4) The results show that isoproterenol acts as a local vasodilatory in the salt gland and also dilatates blood vessels at other sites causing a decrease of blood pressure

Serotonin 10 µg of serotonin i v (*Larus fuscus*) cause a fall in the blood pressure and a corresponding fall in oxygen tension (Fig 4 1) There is no indication of any local effect on the circulation in the salt gland However smaller i a doses produce a decrease of the oxygen tension and little or no change of the blood pressure (Fig 4 2) This indicates that a local vasoconstriction occurs in the gland

Histamine 1—10 μg histamine cause a fall in blood pressure and oxygen (Fig 4 3) but occasionally a rise in oxygen, indicating a vasodilation in the gland is observed (*Larus fuscus*) (Fig 4 4)

Mecholyl (*acetyl beta metylcholine chloride*) Because mecholyl stimulates the secretory mechanism of the salt gland (FANGE *et al* 1958) it is of interest to know how it influences the glandular circulation In the gull 1 to 10 μg mecholyl i v lower the blood pressure and the oxygen tension but have no certain effect on the local circulation (Fig 5 2 3, 4)

Acetylcholine Acetylcholine intra arterially in the gull produces a transient secretion (FANGE *et al* 1958) According to present results i v or i a injection of acetylcholine in the duct cause a fall of blood pressure and available oxygen, but after small i a doses there is a slight initial increase of oxygen tension indicating a vasodilatation of short duration (Fig 5 1)

Discussion

The level of the available oxygen depends on the following factors the external respiration, the blood flow and the metabolic consumption of oxygen If artificial respiration manages to keep the oxygen uptake constant and the local tissue metabolism does not change, the available oxygen probably varies in direct proportion to the blood flow i e depends on the blood pressure and the calibres of the vessels The oxygen increases when the blood pressure goes up and decreases when the latter drops This kind of response which does not reveal any vasomotor reactions has been observed repeatedly during the present work

The vasodilatation during secretion

THESLEFF and SCHMIDT NIELSEN (1962) by direct observation of the gland during nerve stimulation found that the vasodilatation preceeds the electrical response and secretion of the gland According to our recordings the vasodilatation seems to be secondary to the secretion (Fig 2) Probably the vasodilatation is two-phasic caused by 1) impulses from vasodilatory nerve fibres 2) release of a substance from the gland for instance CO_2 or a bradykinin like substance The functional vasodilation during activity of the salt gland ought to be further investigated A certain interpretation of the hitherto made experiments is difficult because nothing is known concerning the magnitude of the glandular metabolism and its changes

The control of the gland

The secretory nerve may be characterized as a parasympathetic nerve The theory of a cholinergic nature of the secretory fibres is supported by the fact that the gland is stimulated to secrete by acetylcholine or mecholyl and the secretion is blocked by atropine (FANGE *et al* 1958) Furthermore the gland (*Larus fuscus*) contains a specific cholinesterase (LUNDIN and FANGE 1962)

The sympathetic fibres to the salt gland are vasoconstrictory and inhibit the secretion These fibres are probably adrenergic The results obtained in the present investigation with pharmacological agents are compatible with the view that the gland is innervated by adrenergic inhibitory and cholinergic stimulatory fibres

The salt gland is influenced both by the autonomic nerves and by adrenocorticosteroid hormones (PHILLIPS HOLMES and BUTLER 1961) This may lead to variable results in physiological experiments with the gland The nervous control of the secretion has so far been studied in gulls only There is the possibility that in certain species the endocrine control of the gland dominates over the nervous or *vice versa*

The basic process of the salt gland cells is an active transport of sodium ions (THESLEFF and SCHMIDT NIELSEN 1962) The presence of corticosteroid hormones is probably necessary for this process to proceed normally As shown by HOKIN and HOKIN (1960), acetylcholine is of biochemical importance for the ion transporting mechanism of the avian salt gland The latter seems to be an organ of choice for investigation of basic mechanisms of nerve and hormone action on glandular cells

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Studies in Neuromuscular Transmission IV

Influence of changes in blood pH and carbon dioxide tension on the effect of succinylcholine

By

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Abstract

GAMSTORP I and E VINNARS *Studies in neuromuscular transmission IV Influence of changes in blood pH and carbon dioxide tension on the effect of succinylcholine* Acta physiol scand 1963 *58* 48—56 — The influence of changes in the arterial blood pH and the partial pressure of carbon dioxide on the effect of succinylcholine was investigated in rabbits The sciatic nerve was stimulated supramaximally and the electric response of the gastrocnemius muscle was recorded the amplitude of the response was used as a measure of the severity of the block. Tachyphylaxis was noted on administration of repeated small doses of succinylcholine but no changes referable to the shift in pH or pCO_2 Changes in pH or pCO_2 had no demonstrable effect on the block caused by a single large dose of succinylcholine The block caused by a continuous infusion of succinylcholine was enhanced by severe respiratory acidosis It was not significantly influenced by mild respiratory acidosis respiratory alkalosis metabolic acidosis or alkalosis or infusion of adrenaline The possibility of these findings being due to a slower hydrolysis of succinylcholine and acetylcholine at a high pCO_2 is discussed

Changes in blood pH and carbon dioxide tension are known to influence the action of neuromuscular blocking agents in clinical anesthesia (DUNDEE 1952, SCURR 1954 GRAY and FENTON 1954) The effects of these changes on the potency of tubocurarine and dimethyl tubocurarine 2 drugs causing a non depolarizing neuromuscular block were studied experimentally by PAYNE

(1958, 1959), JOHANSEN and OSGOOD (1962) and GAMSTORP and VINNARS (1961 b) Succinylcholine, which causes a depolarizing neuromuscular block (THESLEFF 1952) is widely used in clinical anesthesia Changes in the action of depolarizing drugs are more difficult to evaluate since their effect varies from species to species and in different muscles in the same animal (PATON and ZAIMIS 1951 ZAIMIS 1952, 1953) Besides, during constant infusion or repeated injections an originally depolarizing blocking effect may become non depolarizing (ZAIMIS 1952, FOLDES *et al* 1957, CHURCHILL DAVIDSON and CHRISTIE 1959) and weaker (STENBERG and ÖRNDAHL 1957, PAYNE and HOLMDAHL 1959) The following experiments were designed to elucidate the effect of changes in blood pH and carbon dioxide tension on the sensitivity of the rabbit to succinylcholine

Material and methods

Forty six rabbits weighing 2 to 4 3 kg were used Thirty-eight received succinyl choline chloride (Vitrum) (group I II and III) In 8 rabbits (group IV) the influence of respiratory acidosis on the direct motor response was studied The animals in all 4 groups were prepared in the same way This procedure briefly summarized here is described in detail elsewhere (GAMSTORP and VINNARS 1961 b) A tracheal cannula was inserted through a tracheostomy The right femoral artery and vein were catheterized A pair of stainless steel electrodes was applied to the left sciatic nerve and the electric response of the gastrocnemius muscle was recorded through a concentric needle electrode suitably amplified on a cathode ray oscilloscope and photographed The stimulus applied was maintained at supramaximal level throughout the experiment Dextran was infused intravenously in amounts roughly corresponding to the blood loss arterial blood pressure which was measured continuously was maintained at a level above 70 mm Hg The intramuscular temperature was registered it did not change more than 1 5 C during any of the experiments

The frequency and the tidal volume of the artificial respiration were adjusted to maintain a normal blood pH and carbon dioxide tension except when respiratory alkalosis or acidosis was deliberately produced Respiratory alkalosis was induced by doubling or trebling the tidal volume for 10 min Severe respiratory acidosis was produced by administration of 20 % carbon dioxide in oxygen for 10 min in a few experiments 6 5 % carbon dioxide in oxygen was also used to study the effect of mild respiratory acidosis Metabolic alkalosis was produced by intravenous injection of 5—15 meq 0 5 N sodium carbonate solution over 3—8 min Metabolic acidosis was produced by intravenous injection of 4—12 meq 0 5 N hydrochloric acid over 2—8 min

Group I The immediate effect of a small dose of succinylcholine was studied in 9 rabbits Five of the animals were given intra arterial injections each containing 75 μg per kg body weight and 4 received injections of 50 μg per kg The blocking effect of the drug was recorded every 10 sec for the first 1 1/2 min and every 30 sec for the following 3 1/2 min by which time the block had usually disappeared Each animal was given 6 to 8 injections at 20 min intervals In 3 animals the blood pH and carbon dioxide tension were maintained at normal level throughout the experiment In 3 animals respiratory alkalosis and respiratory acidosis were alternately produced for 5 min before and for 5 min after the injections of succinylcholine In 3 animals metabolic alkalosis and metabolic acidosis were produced alternately immediately before the injections Each of these 6 animals received the first 2 injections without previous induction of changes in the blood pH and carbon dioxid tension

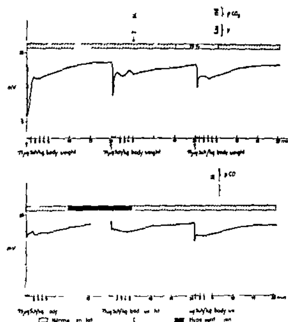


Fig 1 Rabbit no 5 The curve indicates the amplitude of the electric response of the gastrocnemius muscle after supramaximal stimulation of the sciatic nerve The immediate effect of repeated small doses of succinylcholine (Sch)

Group II The late effect of a single dose of succinylcholine was studied in 18 experiments in 11 rabbits The dose used was 0 75 mg succinylcholine per kg body weight When more than one experiment was performed on the same animal the interval between the injections of succinylcholine was at least one hour In 3 experiments the blood pH and carbon dioxide tension were maintained at normal level throughout the experiment In 9 experiments respiratory alkalosis and respiratory acidosis were produced alternately and in 6 experiments metabolic alkalosis and metabolic acidosis were produced alternately in all 15 during the phase of rapidly subsiding block beginning 5 min after the injection of succinylcholine

Group III The block produced by a continuous infusion of succinylcholine was studied in 18 rabbits The rate of the i v infusion was kept constant in each experiment The dose was 20—30 μg per kg per minute At the beginning of the i v infusion each rabbit also received 75 μg per kg intra arterially The actual experiment was not started until 8 to 45 min after the beginning of the infusion by which time the block had become fairly stable In 7 rabbits respiratory alkalosis and severe respiratory acidosis were produced alternately 3 were subjected to 2 periods of acidosis and 1 to 3 periods In addition 5 other rabbits were exposed to 6 5 % carbon dioxide In 5 rabbits metabolic alkalosis and metabolic acidosis were produced alternately one rabbit was exposed to 2 periods of acidosis

Administration of carbon dioxide is known to cause a release of adrenaline (SECHZER *et al* 1960) which may influence the neuromuscular transmission (GOFFART 1957 RUSSELL and SCHULZ 1954 BOWMAN and ZAIMIS 1958) Adrenaline was therefore tested for its effect on the block caused by the continuous infusion of succinylcholine in 6 rabbits The dose of adrenaline was 4 μg per kg per minute given i v at a constant rate for 10 min Four rabbits received another similar dose after an interval of 40—60 min

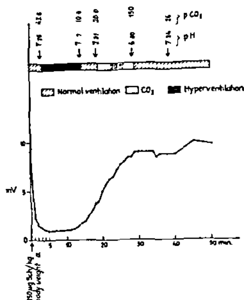


Fig 2 Rabbit no 17 Same as in Fig 1 The late effect of a single large dose of succinylcholine (Sch)

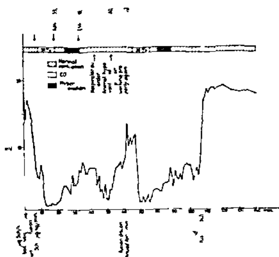


Fig 3 Rabbit no 20 Same as in Fig 1 The effect of succinylcholine (Sch) infused at a constant rate

Edrophonium (0.1 mg/kg) was given i.a. before interrupting the infusion of succinylcholine in 9 of the experiments in group III in order to determine if the drug had caused a block of depolarizing or non-depolarizing type (HOLGS and JOHANSEN 1958)

Group II Total curarization was produced in 8 rabbits by the administration of 3—6 mg tubocurarine per kg body weight, given i.a. over a few minutes Stimulation of the sciatic nerve evoked no response of the gastrocnemius muscle This muscle was then stimulated directly through a needle electrode inserted into the muscle and the

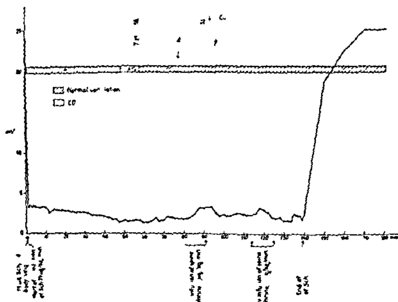


Fig 4 Rabbit no 35 Same as in Fig 1 Succinylcholine (Sch) infused at a constant rate

response was recorded in the same way as described previously The stimulus was maintained at supramaximal level throughout the experiment After 10 min on normal ventilation, the animal was exposed to 20 % carbon dioxide in oxygen for 10 min followed by 10 min normal ventilation The administration of carbon dioxide followed by normal ventilation was repeated in 2 rabbits

The pH of the arterial blood and the partial pressure of carbon dioxide were determined according to the method described by ASTRUP (1956)

Results

Group I Tachyphylaxis was noted in 7 rabbits *i e* the blocking effect of the same amount of succinylcholine diminished with increasing number of injections No changes ascribable to the shift in blood pH and carbon dioxide tension were recorded (Fig 1)

Group II The block disappeared within 15 to 60 min Changes in blood pH and carbon dioxide tension did not influence the steady abatement of the block (Fig 2)

Group III Respiratory alkalosis metabolic alkalosis and metabolic acidosis did not influence the block significantly During severe respiratory acidosis the block increased to diminish again during hyperventilation or normal ventilation This was noted in 9 of 12 experiments performed in 7 rabbits (Fig 3) In one experiment no change at all was recorded in 2 an increase of the block was observed but the experiment could not be completed because movement

of the needle or death of the animal prevented further registration The mild acidosis induced by 6 5 % carbon dioxide had no influence on the block The infusion of adrenaline had no significant effect on the block (Fig 4) The test with edrophonium showed that the block was of the depolarizing type

Group II Severe respiratory acidosis had no influence on the direct muscle response

The administration of 20 % carbon dioxide regularly induced a blood pH below 7 and a partial pressure of carbon dioxide above 130 The metabolic acidosis was of the same magnitude (blood pH was above 7 in only 2 experiments) and the carbon dioxide tension did not alter significantly These results are in accordance with those of earlier experiments in which it was also shown that changes in plasma potassium were too small to influence the neuromuscular transmission (GAMSTORP and VINNARS 1961 a b) Changes in the blood pH and carbon dioxide tension were slight and inconstant during the infusion of adrenaline

Discussion

The enhancing effect of severe respiratory acidosis on the block caused by succinylcholine contrasts with PAYNE s (1958) results In his experiments on cats he recorded the mechanical response of the tibialis anterior muscle after indirect stimulation and found that the block caused by succinylcholine diminished during the administration of 10—20 % carbon dioxide The different experimental conditions may account for the divergent results PAYNE does not state the nature of the block he induced nor does he offer any interpretation of his findings Our results are in agreement with those of DAVIS *et al* (1955) who studied in dogs the effect of succinylcholine on the respiration in dogs before and after exposure to 10—20 % carbon dioxide

The increased sensitivity to succinylcholine during severe respiratory acidosis did not seem due to the release of adrenaline

The effect of severe respiratory acidosis on the unblocked neuromuscular transmission consists of an increase in the stimulus needed to evoke a maximal response (GAMSTORP and VINNARS 1961 a) This cannot however be the explanation for the observed increase of the block since the stimulus was maintained at supramaximal level throughout the experiment

The block caused by tubocurarine was also increased during the administration of carbon dioxide (PAYNE 1958 1959 GAMSTORP and VINNARS 1961 b JOHANSEN and OSGOOD 1962) It would thus appear that the partially blocked neuromuscular junction is sensitive to respiratory acidosis But this explanation is for several reasons unlikely First the tubocurarine block is influenced more by metabolic acidosis than by respiratory acidosis (GAMSTORP and VINNARS 1961 b) the effect can be explained by a shift in the potency of this particular drug due to a change in its ionization following variations of pH (KALOW

1954) Secondly, the block caused by dimethyl tubocurarine differs from that of both tubocurarine and succinylcholine, as it is not influenced significantly by changes in the blood pH or carbon dioxide tension (GAMSTORP and VINNARS 1961 b) Finally, the response to direct electrical stimulation did not change during the administration of carbon dioxide indicating that the excitability of the muscle membrane does not decrease measurably during severe respiratory acidosis

As to the possible influence of pH and carbon dioxide tension on the inactivation rate of succinylcholine and cholinesterase the non enzymatic hydrolysis of succinylcholine decreases with decreasing pH both at 75 C (TAMMELIN 1953) and at 21 C (FRASER 1954) In the intact animal succinylcholine is rapidly hydrolysed by plasma cholinesterase (GLICK 1941, CASTILLO and DE BEER 1950) The urinary excretion of the drug is negligible (FOLDES and NORTON 1954 FOLDES VANDEVORT and SHANOR 1955) Cholinesterase has optimal effect at pH 8 (JANSZ and COHEN 1962) and is most stable at pH 7 4 (*CHAUDHURI 1949*) *it is gradually inactivated at decreasing pH* (*AUGUSTINSSON* 1948) Thus, both the non enzymatic and the enzymatic break down of succinylcholine may decline during acidosis The enzymatic hydrolysis of acetylcholine is also influenced by changes in pH and carbon dioxide tension (TENNEY 1960) If the pH is lowered by an increase of the carbon dioxide tension the effect of acetylcholine will increase this effect has been ascribed to the change in pH (BEAN and SIDKY 1958) However if the carbon dioxide tension is kept constant acetylcholine becomes less effective if the pH is lowered below 7, if the pH, on the other hand, is kept constant, the effect of acetylcholine increases with the carbon dioxide tension (MARTI STAMM and BUCHER 1956, STAMM 1957) Since an accumulation of acetylcholine at a neuro muscular junction partially blocked by a depolarizing drug will increase the block this factor may explain why respiratory acidosis but not metabolic acidosis enhances the block caused by succinylcholine

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Acta physiol scand 1963 58 57—67

From the Institute of Physiology University of Lund Sweden

Location, Course, and Characteristics of Uncrossed and Crossed Ascending Spinal Tracts in the Cat

By

B HOLMQVIST and O OSCARSSON

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Abstract

HOLMQVIST B and O OSCARSSON *Location course and characteristics of uncrossed and crossed ascending spinal tracts in the cat* Acta physiol scand 1963 *58* 57—67 — Discharges evoked in coarse fibred ascending spinal tracts on stimulation of muscle and skin nerves in the hindlimbs and on stimulation of lumbar sacral and caudal dorsal roots were recorded from dissected fascicles of the cord at L1 and C3 Tracts in the dorsal part of the lateral funiculus are monosynaptically activated only from ipsilateral afferents and tracts located ventrally thereof only from contralateral afferents (exceptional findings on stimulation of the most caudal roots are related to the crossing of primary afferents in these caudal segments) It is concluded that the former tracts are uncrossed and the latter crossed at the spinal level — There is no obvious segmental lamination of fibres within the areas of uncrossed and crossed tracts Individual tracts occupy relatively fixed and well defined areas independent of their level of origin There is a marked dorsal shift of the dorsal and ventral spinocerebellar tracts during their ascent in the cord

The general organization of coarse fibred ascending spinal tracts was recently investigated in experiments on the phalanger and rabbit (MAGNI and OSCARSSON 1962 b) These experiments were limited to a study of the organization of tracts activated from hindlimb afferents in the lumbar segments they showed that

a) tracts ascending in the dorsolateral funiculus are activated mono- and polysynaptically only from ipsilateral nerves

b) tracts ascending in the ventral quadrant (ventrolateral funiculus + ventral funiculus) are activated monosynaptically only from contralateral nerves and polysynaptically from both ipsilateral and contralateral nerves

These observations and histological evidence that primary afferents terminate almost exclusively on the ipsilateral side suggest that the latter but not the former tracts ascend after having crossed the midline of the cord

The present investigation has confirmed, in the cat the previous findings made on the phalanger and rabbit In addition the observations have been extended to tracts activated on stimulation of dorsal roots belonging to lumbar, sacral and caudal segments, and to the change in position of tracts activated from hindlimb afferents during their ascent in the cord The location and characteristics of tracts activated from forelimb afferents are described in a separate paper (HOLMQVIST, OSCARSSON and UDDENBERG 1963)

Methods

The experiments were done on cats under light pentobarbitone anaesthesia The animals were artificially ventilated and flaxedil was given to prevent reflexes The body temperature was kept between 36 and 39° C

In some cats a laminectomy including all lumbar vertebrae was made and the cord transected at the lower Th 13 level The dorsal funiculi were removed down to slightly below the first lumbar roots The remaining part of the cord was divided along the midline and the cord halves split longitudinally into smaller subdivisions (called fascicles below *cf* Fig 1—4) and recorded from as described by LAPORTE LUNDBERG and OSCARSSON (1956) Ingoing volleys were monitored at the dorsal root entrance by triphasic recording In other cats the second cervical vertebra was delaminated. The cord was transected at the level of the second cervical roots and the dorsal funiculi dissected free but left in continuity with the cord at the caudal end of C3 The cord halves were split into fascicles of various sizes and recorded from. The hamstring sural and superficial peroneal (except muscle branches) nerves were dissected for stimulation bilaterally At the end of some experiments the dorsal roots of the lower lumbar sacral and upper caudal segments were severed bilaterally and mounted on stimulating electrodes The nerves and exposed parts of the cord were covered with warm mineral oil

The stimulating and recording technique has been described before (LAPORTE *et al* 1956 MAGNI and OSCARSSON 1962 a) The stimulus strength was 15—30 times that evoking a barely perceptible ingoing volley Two double beam oscilloscopes were used one usually displayed the ingoing volley and the early part of the ascending discharge on a fast time base the other displayed the ascending discharge on a slow time base. The records were formed by photographic superposition of 15—20 faint traces

The cross sections of the fascicles recorded from were assessed after fixation of the removed cord segment by inspection through a binocular preparation microscope The circumference of the cord and the distances between the various sections were measured Additional controls were obtained from serial sections of the region where the fasciculi were in continuity with the intact cord

Results

1 Organization of tracts at the first lumbar segment

In the experiment of Fig 1 the spinal cord half was split into four fascicles (I—IV), the two dorsal ones corresponding to the dorsolateral funiculus and the two ventral ones to the ventral quadrant Each set of traces shows the

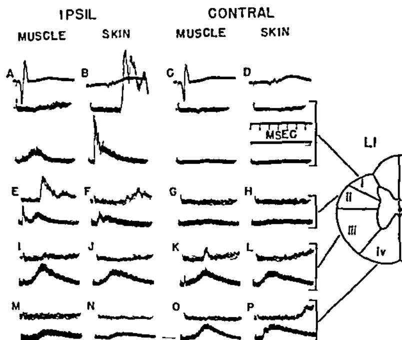


Fig 1 Discharges recorded at the first lumbar segment from tracts activated by stimulation of ipsilateral and contralateral muscle (hamstring) and skin (superficial peroneal) nerves The records were obtained from fascicles i—iv as indicated The ingoing volley was recorded triphasically from the dorsal funiculus at the upper L7 level and is shown in A—D (upper traces) The pairs of traces show the discharges recorded simultaneously on a fast and slow time base The distance between the two recording sites was 6 5 cm

ascending discharge recorded from the indicated fascicle at two speeds The top traces show the ingoing volley recorded triphasically from the dorsal root entrance (*cf* Methods) In the dorsomedial fascicle (i) a large monosynaptic response was evoked on stimulation of the ipsilateral skin nerve (B) in the dorsolateral fascicle (ii) a large monosynaptic discharge was produced by stimulation of ipsilateral group I muscle afferents (E) The former discharge was due to activity in the dorsomedial cutaneous tract described by LUNDBERG and OSCARSSON (1961) the latter to activity in the dorsal spinocerebellar tract (DSCT) (*cf* LUNDBERG and OSCARSSON 1961) Stimulation of contralateral nerves evoked no discharge in the dorsomedial fascicle (C D) and only a trace of late activity in the dorsolateral fascicle (G H)

On the other hand the two ventral fascicles contained tracts dominated by effects from contralateral nerves In the ventrolateral fascicle (iii) the spike like discharge (K) was due to activity in the ventral spinocerebellar tract (VSCT) elicited by a volley in contralateral group I muscle afferents (OSCARSSON 1956) The small monosynaptic discharge elicited by ipsilateral group I

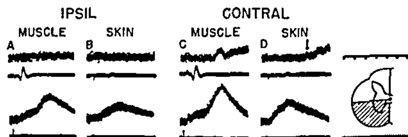


Fig 2 Discharges recorded at the first lumbar segment from tracts ascending in the ventral fascicle (hatched area in diagram) on stimulation of ipsilateral and contralateral muscle (hamstring) and skin (sural) nerves Triphasic recording of the ingoing volley in the dorsal roots and recording of the ascending discharge were made simultaneously on a fast (upper pair of traces) and slow (lower pair of traces) time base The arrow indicates monosynaptic response evoked from the skin nerve Time scales in msec The distance between the recording sites was 8 cm

muscle afferents (I) was due to activity in a small part of the DSCT included in the dissected fascicle The ventromedial fascicle (iv) displayed no distinct monosynaptic responses (M—P) The late activity was larger when evoked from contralateral nerves (compare M N with O P) Only sometimes did skin nerve volleys produce a distinct monosynaptic discharge in the ventral part of the cord One example is shown in Fig 2 The cord was split slightly below the level of the central canal This eliminated completely any monosynaptic responses from the ipsilateral nerves (A, B) The discharges evoked from the ipsilateral nerves appeared on activation of group II muscle afferents and low threshold cutaneous afferents the long latency indicates polysynaptic transmission On the other hand monosynaptic discharges were evoked from both muscle and skin afferents in contralateral nerves Group I muscle afferents were responsible for the monosynaptic discharge in the VSCT (C) and the cutaneous afferent volley evoked a discharge (D, indicated by arrow) with a similar delay

Our observations show that the organization in the cat is similar to that previously reported for the phalanger and rabbit (MAGNI and OSCARSSON 1962 b) In all three animal species the dorsolateral funiculus in the lumbar region contains tracts activated only from ipsilateral nerves and the ventral quadrant, tracts monosynaptically activated only from contralateral nerves and polysynaptically activated predominantly from contralateral nerves However, the present experiments suggest also that the late activity evoked from contralateral nerves increases gradually from dorsal to ventral regions of the cord (Fig 1)

The organization described above applies to tracts activated from hindlimb afferents Two reasons made it of interest to study the discharges evoked on stimulation of dorsal roots First, the afferents in the lower sacral roots are

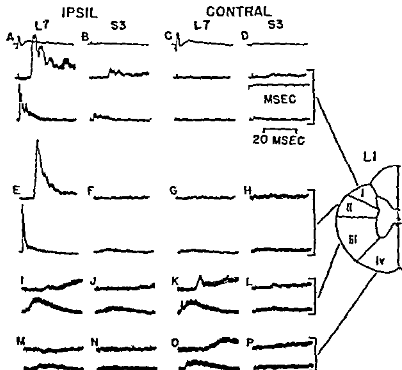


Fig 3 Discharges recorded at the first lumbar segment from tracts activated by stimulation of ipsilateral and contralateral L7 and S3 dorsal roots. From the experiment also shown in Fig 1 The records were obtained from fascicles i—iv as indicated. The ingoing volley was recorded triphasically from the dorsal funiculus at the L7 level (upper traces in A—D) The pairs of traces show the discharges recorded simultaneously on a fast and slow time base The distance between the two recording sites was 6 5 cm

known to send branches across the midline of the cord which contrasts to the almost exclusively ipsilateral termination of dorsal root fibres at other levels (SPRAGUE 1958) Secondly there was the possibility that afferents innervating midline structures had central connections different from those of afferents innervating the limbs

Dorsal roots down to and sometimes including S2 evoked discharges according to a pattern similar to that observed for discharges evoked from hind limb nerves In Fig 3 stimulation of the ipsilateral L7 dorsal root produced a large discharge (A) in the dorsomedial fascicle (i) but no discharge was evoked from the contralateral root (C) In the dorsolateral fascicle (ii) there was a large ipsilateral discharge (E) but only a barely visible late contralateral discharge (G) comparable to that evoked from hindlimb nerves earlier in the same experiment (Fig 1 G and H) The discharges (I K M O) evoked

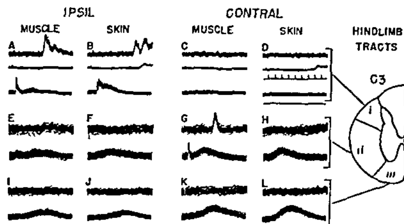


Fig 4 Discharges recorded at the third cervical segment from tracts activated by stimulation of ipsilateral and contralateral muscle (hamstring) and skin (superficial peroneal) nerves in the hindlimbs The records were obtained from fascicles i—iii as indicated The upper and lower traces show the discharges recorded simultaneously on a fast and slow time base Middle traces in A—D show ascending volleys recorded from the dissected dorsal funiculi at C3 Time scales in msec Distance from stimulating electrode on hamstring nerve to C3 about 37 cm.

in the two ventral fascicles (iii iv) also followed the pattern of discharges evoked from limb nerves

Stimulation of more caudal roots evoked discharges which in two respects differed from the pattern described above

a) In the dorsomedial fascicle a distinct monosynaptic discharge about one third in size of the ipsilateral discharge was produced by stimulation of the contralateral S3 root (Fig 3 D) A small monosynaptic discharge was also evoked contralaterally in the dorsolateral fascicle and was possibly due to activity in the same category of fibres (H) The contralateral discharge in the dorsolateral funiculus should be connected with the contralateral termination of primary afferents in the dorsal and ventral horns of the lower sacral segments (Sprague 1958) and with the finding of crossed monosynaptic connections to motoneurones in these segments (Curtis Krnjević and Miledi 1958 Frank and Sprague 1959) Hence the fibres ascending in the dorsolateral funiculus and activated from contralateral afferents in the lower sacral and caudal roots may originate from ipsilateral cell bodies

b) There was never any monosynaptic response from the lower sacral and caudal roots corresponding to activity in the DSCT (F) whereas a monosynaptic discharge presumably representing activity in the VSCT was regularly seen (L) This might suggest that group I muscle afferents from the lower sacral and caudal roots do not reach the Clarke s column cells However degeneration in Clarke s column has been described after section of the S3—S4 dorsal roots (Grant and Rexed 1958)

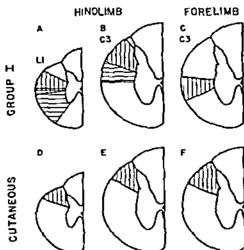


Fig 5 Location of tracts monosynaptically activated from group I muscle afferents (A—C) and of the dorsomedial cutaneous tract (D—F) The areas occupied by tracts activated from ipsilateral nerves are indicated by vertical hatching and the areas occupied by tracts activated from contralateral nerves by horizontal hatching A B D and E show location of tracts activated from hindlimb nerves at the indicated segments C and F location of tracts activated from forelimb nerves at the C3 segment The latter tracts have been described in a separate paper (Holmqvist *et al* 1963)

2 Spinal course of certain tracts

There are several anatomical reports suggesting a dorsal shift of certain tracts during their ascent in the cord (*e g* Walker 1940 Yoss 1952 1953 Smith 1957) We have now confirmed these observations by recording from dissected fascicles at the level of the third cervical segment

The experiment in Fig 4 will be shown as an example Three fascicles were recorded from on stimulation of muscle and skin nerves in the hindlimbs The monosynaptic DSCT discharge (A) evoked from group I muscle afferents was almost completely confined to the dorsal fascicle (i) and so was the monosynaptic discharge evoked in the dorsomedial cutaneous tract (B) It is concluded that the DSCT has a much more dorsal position at the cervical than at the lumbar level. As could be expected no discharges were elicited by volleys in the contralateral nerves (C D) The monosynaptic VSCT discharge (G) evoked by stimulation of contralateral group I afferents was recorded in the lateral fascicle (ii) together with late activity produced by impulses in skin and high threshold muscle afferents (E—H) In the ventral fascicle (iii) no monosynaptic discharges could be discerned and contralateral nerves were more effective in evoking large discharges than ipsilateral nerves (I—L) It should be noted that the discharges in the ventral fascicle had a longer latency and later summit than the late discharges in the lateral fascicle At the first lumbar segment there was no appreciable difference in this respect between for example fascicles (iii) and (iv) in Fig 1 This suggests that the active fibres in the ventral fascicle at C3 had a lower conduction velocity than the active fibres in the lateral fascicle

The large number of variously dissected fascicles (27 in the first lumbar and 32 in the third cervical segment) made it possible to determine fairly

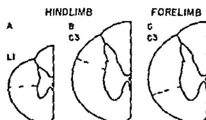


Fig 6 Areas occupied by uncrossed (dorsally of broken line) and crossed tracts (ventrally of broken line) at indicated segments A and B refer to tracts activated from hindlimb nerves C to tracts activat d from forelimb nerves

accurately the position of some tracts Fig 5 shows the areas occupied by tracts activated from group I muscle afferents (A—C) and the areas occupied by the dorsomedial cutaneous tract (LUNDBERG and OSCARSSON 1961) (D—F) The figure includes observations on tracts activated from forelimb afferents which have been described in a separate paper (HOLMQVIST *et al* 1963)

A and B, Fig 5, show the areas occupied by the dorsal (vertical hatching) and ventral (horizontal hatching) spinocerebellar tract In the lumbar region DSCT occupies the dorsolateral corner of the cord and extends ventrally somewhat past the horizontal plane going through the central canal In the cervical region it has changed to a more dorsomedial position though there is still a small medial area relatively devoid of DSCT fibres The VSCT is located in the ventrolateral corner of the cord in the lumbar region it extends approximately from the level of the central canal to the ventral root exit (Fig 5, A horizontal hatching) In the cervical region it has a much more dorsal position it occupies approximately the same area as DSCT does in the lumbar region (B) The anatomical separation of the DSCT and VSCT was if anything, better at the cervical than at the lumbar level

It is interesting to compare our results with the histological analysis of ascending tracts made by SMITH (1957) on human material She observed that the superficial coarse fibres in the ventrolateral region of the lumbar cord shifted to a position in the dorsolateral funiculus at the upper cervical segments whereas thinner and more deeply situated fibres in the ventrolateral region of the lumbar cord remained ventrally Her results are in good agreement with our finding of a dorsal shift of the VSCT The late discharge with a long latency found in the ventral fascicle (III) (Fig 4 K, L) may be due to activity in the thinner tract fibres mentioned by SMITH A dorsal shift of the DSCT and VSCT was recently described by GRANT (1962)

Group I muscle afferents in forelimb nerves activate only one ascending spinal tract (HOLMQVIST *et al* 1963) This tract is uncrossed originates from cells above Clarke s column and is not homologous with either DSCT or VSCT Its position is shown in Fig 5 C

Fig 5 D—F shows the area occupied by the dorsomedial cutaneous tract (LUNDBERG and OSCARSSON 1961) The component activated from hindlimb afferents is medial of the DSCT at the lumbar level (D) but largely overlaps

the area of this tract at the cervical level (E) The forelimb component of the cutaneous tract extends slightly more ventrally than the hindlimb component at the cervical region (compare E and F)

Discussion

The present findings substantiate the suggestion made in a previous paper (MAGNI and OSCARSSON 1962 b) that uncrossed ascending spinal tracts occupy the dorsal part of the lateral funiculus and crossed tracts the ventral part of this funiculus and the ventral funiculus There is convincing histological evidence that primary afferents make synaptic contacts almost exclusively with ipsilateral cell bodies though exceptions to this rule occur in the first cervical and lower sacral segments (SCHIMERT 1939 ESCOLAR 1948, LIU 1956 SPRAGUE 1958) Our observation that dorsally located tracts receive monosynaptic excitation only from ipsilateral afferents and ventrally located tracts only from contralateral afferents indicates that the former tracts are uncrossed and the latter crossed at the spinal level The exceptional finding of a contralateral discharge in dorsally located fibres on stimulation of lower sacral and caudal roots is reasonably explained by the crossing of primary afferents in these segments (SPRAGUE 1958)

The areas occupied by uncrossed and crossed tracts vary in extent at different levels of the cord and depend also on the segmental origin of the tracts A dashed line is drawn between uncrossed and crossed tracts in Fig 6 In the lumbar region the uncrossed tracts occupy approximately the dorsal half of the lateral funiculus (A) In the cervical region the uncrossed hindlimb tracts are located in the dorsal third and the uncrossed "forelimb tracts in the dorsal two thirds of the lateral funiculus (B C) (*cf* HOLMQVIST *et al* 1963)

Some authors but not others have found a segmental lamination of ascending fibres in the dorsolateral funiculus and ventral quadrant of the cord with fibres originating from caudal levels located most dorsally in the respective area (*e g* SHERRINGTON and LASLETT 1903 BECK 1927 WALKER 1940 MORIN SCHWARTZ and O LEARY 1951 YOSS 1952 1953 VAN BEUSEKOM 1955) The question if a segmental lamination exists can be given a more distinct form by asking if there is a lamination within the areas containing uncrossed and crossed tracts respectively A crude lamination might be suggested by the fact that the uncrossed forelimb tracts extend further ventrally than the uncrossed hindlimb tracts and *vice versa* the crossed hindlimb tracts extend further dorsally than the crossed forelimb tracts (Fig 6 B and C) However this cannot be taken to indicate a true dorsoventral lamination as the uncrossed forelimb tracts overlap the uncrossed hindlimb tracts and correspondingly the crossed hindlimb tracts overlap the crossed forelimb tracts Furthermore individual tracts seem to occupy relatively fixed and well defined areas independent of their level of origin This can be illustrated by

examples from the group of uncrossed tracts The forelimb component of the dorsomedial cutaneous tract (LUNDBERG and OSCARSSON 1961) is located in the most dorsal part of the lateral funiculus whereas the forelimb tract activated from group I muscle afferents has a relatively ventral position it occupies the middle third of this funiculus (Fig 5, C and F) Both forelimb and hindlimb components of the dorsomedial cutaneous tract are medial of, but overlap partly the (hindlimb) DSCT (Fig 5, B E and F) However, our observations do not exclude a segmental lamination within individual tracts (cf SHERRINGTON and LASLETT 1903 HYNDMAN and EPPS 1939, YOSS 1952 1953 GRANT 1962) A coarse lamination of this type is indicated by the more ventral extent of the area occupied by the forelimb component of the dorsomedial cutaneous tract than of the area occupied by the hindlimb component (Fig 5 E and F)

Some of the investigated tracts shifted dorsally during their ascent in the cord which is in agreement with a number of anatomic investigations (*e g* YOSS 1952 1953 SMITH 1957 GRANT 1962) A dorsal shift was clearly established for the DSCT and VSCT and it should be stressed that the latter tract in the cervical region has a relatively dorsal position (Fig 5, B) In the C3 segment a lesion in the dorsal half of the lateral funiculus would interrupt not only DSCT but also VSCT This necessitates a reconsideration of certain earlier experiments in which lesions of this type were made for supposed selective interruption of the DSCT (*e g* MORIN and HADDED 1953 MORIN and GARDNER 1953)

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From the Institute of Physiology, University of Lund Lund Sweden

Organization of Ascending Spinal Tracts Activated from Forelimb Afferents in the Cat

By

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Received 12 October 1962

Abstract

Holmqvist B O Oscarsson and N Uddenberg *Organization of ascending spinal tracts activated from forelimb afferents in the cat* Acta physiol scand 1963 *58* 68—76 — Discharges evoked in coarse fibred ascending spinal tracts on stimulation of muscle and skin nerves in the forelimbs were recorded from dissected fascicles of the cord at C3 The general organization of tracts activated from fore limb afferents is similar to that of tracts activated from hindlimb afferents uncrossed tracts are located in the dorsal part of the lateral funiculus and crossed tracts ventrally thereof In the cervical region the uncrossed tracts activated from forelimb afferents occupy a larger area than those activated from hindlimb afferents — Group I muscle afferents activate only one ascending tract It is uncrossed and ascends in the middle third of the lateral funiculus This tract is not identical with either the dorsal or the ventral spinocerebellar tract It is suggested that these two tracts lack forelimb components — The dorsomedial tract activated from low threshold cutaneous afferents and terminating in the lateral cervical nucleus seems to originate from both hindlimb and forelimb levels of the cord

There is now considerable knowledge about the organization of coarse fibred ascending spinal tracts activated from hindlimb afferents in the cat The properties of the spinocerebellar tracts and several other ascending systems are known in detail (cf Lundberg and Oscarsson 1960 1961 1962a b) On the other hand, there is little information about the tracts activated from forelimb afferents The present investigation is introductory to a series of studies planned to explore the organization of these tracts It will be shown below that group I muscle afferents in forelimb nerves do not activate any forelimb component of the dorsal or ventral spinocerebellar tract (DSCT or VSCT) However, a

volley in these afferents evoke a discharge in a spinal tract unknown from previous investigations concerned with tracts ascending from the lumbar enlargement of the cord

The present investigation is also closely related to previous studies on the general organization of ascending spinal tracts in mammals (MAGNI and OSCARSSON 1962b HOLMQVIST and OSCARSSON 1963) Recording of discharges in tracts activated from hindlimb afferents suggests that uncrossed tracts are located in the dorsal part of the lateral funiculus and crossed tracts ventrally thereof in the lateral and ventral funiculi The present observations show that uncrossed and crossed forelimb tracts have similar locations in the cord

Methods

The experiments were done on cats under light pentobarbitone anaesthesia Some of them were performed at the same time as those described in a previous paper (HOLMQVIST and OSCARSSON 1963) The animals were artificially ventilated and flaxedil was given to prevent reflexes The body temperature was kept between 36 and 38 C

The second cervical vertebra was delaminated The cord was transected at the level of the second cervical roots and the dorsal funiculi dissected free but left in continuity with the cord at the caudal end of C3 in order to permit monitoring of ingoing volleys from forelimb nerves The remaining part of the spinal cord was divided along the midline and the cord halves split into smaller subdivisions (called fascicles below cf Fig 1) and recorded from as described by LAPORTE LUNDBERG and OSCARSSON (1956) In some cases the laminectomy was extended at the end of the experiment for making lesions or for recording the ingoing volley triphasically at the dorsal root entrance or monophasically from the cut dorsal root

The deep radial nerve (dorsal interosseus) and the superficial radial nerve were dissected bilaterally and mounted on stimulating electrodes In some experiments also hindlimb nerves were dissected for stimulation The stimulating and recording technique has been described before (LAPORTE *et al* 1956 MAGNI and OSCARSSON 1962a HOLMQVIST and OSCARSSON 1963) When not especially stated the stimulus strength was 15—30 times that evoking a barely perceptible ingoing volley The cross-sections of the fascicles recorded from were assessed as described by HOLMQVIST and OSCARSSON (1963)

Results

I General organization of forelimb tracts

Certain general rules have been recognized in the organization of ascending spinal tracts activated from hindlimb afferents (MAGNI and OSCARSSON 1962b HOLMQVIST and OSCARSSON 1963) Tracts in the dorsal part of the lateral funiculus are mono and polysynaptically activated only from ipsilateral nerves whereas more ventrally located tracts are monosynaptically activated only from contralateral nerves and polysynaptically from both ipsilateral and contralateral nerves We have now shown that these rules apply also to tracts activated from forelimb afferents

Three dissected fascicles (i—iii see diagram) were recorded from in the experiment of Fig 1 The upper and lower traces in each set of records show the mass discharge in the indicated fascicle at two speeds The middle trace in

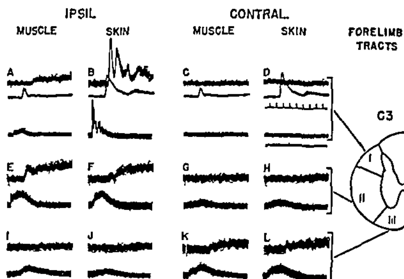


Fig 1 Discharges recorded at the third cervical segment from tracts activated by stimulation of ipsilateral and contralateral muscle (deep radial) and skin (superficial radial) nerves in the forelimbs The records were obtained from the dissected fascicles i—iii as indicated The upper and lower traces show the discharges on a fast and slow time base Middle traces in A—D show ascending volleys recorded from the disssected dorsal funiculi at C3 Time scales in msec Distances stimulating electrodes on the nerves — C7 dorsal root entrance 11 5 cm C7 dorsal root entrance — recording place 4 5 cm.

the upper row of records shows the ingoing volley recorded from the dissected dorsal funiculi In the dorsal fascicle (i) discharges were evoked by stimulation of ipsilateral muscle and skin nerves but not by stimulation of contralateral nerves (A—D) In the lateral fascicle (ii) large discharges initiated by monosynaptic components were evoked by ipsilateral volleys (E F) Stimulation of contralateral nerves produced only a small late activity (G H) In the ventral fascicle (iii) monosynaptic discharges were elicited by muscle and cutaneous afferent volleys in contralateral nerves (K L) The discharges evoked from ipsilateral nerves appeared on activation of group II muscle afferents and low threshold skin afferents the long latency indicates that the transmission was polysynaptic (I, J)

This and other experiments show that the general organization of hindlimb and forelimb tracts is similar However the tracts activated monosynaptically from ipsilateral nerves occupy at the C3 level the dorsal two thirds of the lateral funiculus when originating from the cervical enlargement of the cord and only the dorsal one third when originating from the lumbar enlargement (cf Holmqvist and Oscarsson 1963)

2 Discharges evoked from muscle afferents

The ingoing volley in the muscle nerve (deep radial) was analysed by monophasic recording from the severed C7 dorsal root The group I volley reached a maximum at 1 9 times threshold in the experiment illustrated in Fig 2 A—H The group II volley

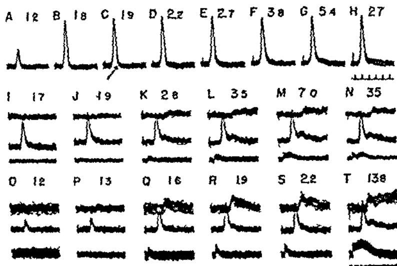


Fig 2 Afferent fibre groups responsible for the discharges evoked in tracts activated by stimulation of the ipsilateral muscle (deep radial) nerve in the forelimb From the experiment also shown in Fig 1 I—N show discharges recorded from the dorsal fascicle (Fig 1 i) and O—T discharges recorded from the lateral fascicle (Fig 1 ii) Upper and lower traces show the discharges recorded simultaneously on a fast and slow time base Middle traces show ingoing volleys recorded from dissected dorsal funiculi at C3 Stimulus strength relative to threshold of ingoing volley indicated on each record Record Q is retouched to show the volley in the dorsal funiculus

A—H show the volley evoked by stimulation of the deep radial nerve and recorded at the end of the experiment monophasically from the cut C7 dorsal root. Stimulus strength relative to threshold indicated on each record. Arrow indicates small potential due to activation of low threshold group II afferents Time scales in msec. For distances see Fig 1

was barely perceptible at the same strength (C indicated by arrow) it became clearly visible at higher strengths of stimulation (D—H) In other experiments the group I volley became maximal at 1 4—2 0 times threshold and the group II afferents had a threshold at the relative strengths of 1 3—2 0 i e at a strength just below that evoking a maximal group I volley These values are comparable with those obtaining for group I and II muscle afferents in hindlimb nerves (BROCK ECCLES and RALL 1951 ECCLES ECCLES and LUNDBERG 1957b ECCLES and LUNDBERG 1959)

Stimulation of group I muscle afferents did not evoke any activity in the dorsal part of the lateral funiculus in the classical region of the DSCT The small discharge in the dorsal fascicle (i) illustrated in Fig 1 A was due to a volley in group II muscle afferents as demonstrated in Fig 2 I—N from the same experiment The early part of this discharge appeared at 1 9 times threshold and reached a maximum at 7 0 times threshold The latency was so short as to indicate monosynaptic transmission (the conduction distances are given in Fig 1)

Similar observations showed that the small early discharge elicited from contralateral muscle nerves in the ventral fascicle (iii) (Fig 1 K) ·

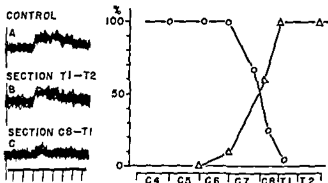


Fig 3 Three experiments made in order to determine the segmental level of the cell bodies of the group I activated forelimb tract A—C show discharges recorded from the dissected lateral funiculus at C3 on stimulation of the ipsilateral deep radial nerve at slightly supramaximal group I strength A was obtained before any transection of the spinal cord B after transection between the first and second thoracic segment and C after transection between the eighth cervical and first thoracic segment Time scale in msec The diagram shows two experiments In one experiment the dorsal funiculi were transected at successively more caudal levels (open circles) in the other the lateral funiculus was transected at successively more rostral levels (triangles) while the mass discharge was recorded from the dissected lateral funiculus at C3 on stimulation of the ipsilateral deep radial nerve at slightly supramaximal group I strength Ordinate amplitude of monosynaptic discharge in per cent of control value Abscissa segmental level of transection following which the mass discharge was recorded and measured The fourth cervical to second thoracic segments are indicated on the horizontal scale See text

to a volley in group II muscle afferents it had a latency which indicates monosynaptic transmission

The large monosynaptic discharge in the lateral fascicle (ii) (Fig 1 E) is of special interest as it represents the only activity in ascending spinal tracts that was evoked by a volley in group I muscle afferents from the forelimb nerves In the experiment of Fig 2 the discharge appeared at a strength of 1 3 times threshold (O P) and grew to a maximum at 1 9 (R) In the various experiments the threshold of this discharge varied between 1 2 and 1 4 and the maximum was obtained at, or slightly below maximum for the group I volley The relatively high threshold may signify the need for spatial summation or it may indicate that *high threshold group I afferents alone contribute excitatory action* The threshold of 1 2 to 1 4 is similar to the threshold of group Ib afferents in hind limb nerves (BRADLEY and ECCLES 1953, ECCLES ECCLES and LUNDBERG 1957a) This might suggest that the forelimb tract receives excitation from Ib but not Ia afferents

It was important to obtain anatomical data concerning the group I activated forelimb tract for a comparison with the likewise group I activated dorsal and ventral spinocerebellar tracts Fig 3 illustrates three experiments made in order to determine the level of the cells of origin The mass discharge was not appreciably reduced after transection of the cord between Th1 and Th2 but diminished following a transection between C8 and Th1 (Fig 3, A—C) The

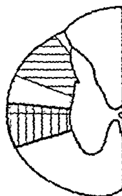


Fig 4 Location of the group I activated forelimb tract at the lower C3 level. The spinal cord sector containing this tract is indicated by vertical hatching For comparison the sectors are shown which contain the dorsal (horizontal hatching) and ventral (stippled) spinocerebellar tract (*cf* HOLMQVIST and OSCARSSON 1963)

decrease after the second lesion was partly due to interruption of the primary afferents which enter the cord through the C7 C8 and Th1 dorsal roots It is concluded that the discharge was not relayed in cells located caudally of the dorsal root entrance and activated by descending branches in the dorsal funiculus as might be suggested from the anatomical observations of LIU (1956 *cf* GRANT and REXED 1958) The level of the synaptic relay was then determined by transection of the dorsal funiculus (interruption of presynaptic fibres) at successively more caudal levels (open circles) and by transection of the lateral funiculus (interruption of postsynaptic axons) at successively more rostral levels (triangles) while the effect on the mass discharge was watched by recording from the lateral funiculus (Fig 3 diagram) The experiments show that the relay occurs at or slightly above the level of the dorsal root entrance

The location of the group I activated forelimb tract was assessed from recordings made from the large number (32) of variously dissected fascicles in the C3 segment The tract ascends in the middle third of the lateral funiculus ventral of but partly overlapping the area occupied by the VSCT at this level (Fig 4)

3 Discharges evoked from cutaneous afferents

The large discharge evoked in the dorsal fascicle (i) in Fig 1 B had similar characteristics as the discharge in the dorsomedial cutaneous tract described by LUNDBERG and OSCARSSON (1961) it had a similar location in the cord was monosynaptically elicited by a barely visible ingoing volley and had a repetitive character It is suggested that this discharge was evoked in the forelimb component of the dorsomedial cutaneous tract

A small early discharge appeared in the lateral fascicle (ii) (Fig 1 F) at a strength of about 1 5 times threshold The latency indicates a monosynaptic linkage The small contralateral monosynaptic discharge evoked in the ventral fascicle (iii) (Fig 1 L) had only a slightly longer latency (0 1 msec) than the large ipsilateral discharge in the dorsal fascicle (B)

Discussion

This investigation has shown that the general organization of coarse fibred ascending spinal tracts activated from forelimb afferents is similar to that of tracts activated from hindlimb afferents tracts in the dorsal part of the lateral funiculus receive monosynaptic excitation exclusively from ipsilateral afferents and tracts located ventrally thereof exclusively from contralateral afferents It has previously been argued that the former tracts are uncrossed and the latter crossed at the spinal level (MAGNI and OSCARSSON 1962b, HOLMQVIST and OSCARSSON 1963)

Except for these general findings our experiments demonstrated considerable differences in the organization of tracts originating from hindlimb and fore limb levels of the cord The group I muscle afferents in hindlimb nerves activate exclusively two ascending tracts the dorsal and ventral spinocerebellar tract (DSCT and VSCT) (LUNDBERG and OSCARSSON 1960 1962a) Only one spinal tract was activated from group I afferents in forelimb nerves This tract is distinguished from the DSCT and VSCT by its anatomical characteristics It differs from the DSCT in arising from cell bodies located rostrally of Clarke s column and having a ventral position in the cord and from the VSCT in being uncrossed Our observations are in agreement with recent anatomical observations on the spinocerebellar tracts GRANT and REXED (1958) showed that the cells in CLARKE s column are innervated exclusively by afferents in the dorsal roots caudally of the first thoracic segment Terminal degeneration in the cerebellar cortex is limited to the hindlimb areas not only when the spinocerebellar tracts are severed in the thoracic region but also when they are severed in the cervical region (GRANT 1962b) Hence anatomical and physiological experiments indicate that the DSCT and VSCT forward information from the hindlimbs but not the forelimbs

It may be asked whether the type of information conveyed by the spinocerebellar tracts reaches the cerebellum through other pathways when originating from the forelimbs The cuneocerebellar tract terminates in the cerebellum in a manner resembling the termination of the DSCT but reaches exclusively the forelimb areas (GRANT 1962a) It has recently been shown that this tract contains proprioceptive and exteroceptive components similar to those in DSCT and concluded that the two tracts are largely equivalent as channels of information from the forelimbs and hindlimbs respectively (HOLMQVIST, OSCARSSON and ROSÉN 1963)

The possibility should be considered that the group I activated forelimb tract is a functional homologue to VSCT However the observations of GRANT (1962b) that transection of the lateral funiculus in the cervical region does not result in terminal degeneration outside the hindlimb areas of the cerebellar cortex suggest that this tract does not reach the cerebellum uninterrupted by

synaptic relays A further discussion of this tract should be postponed until its termination and detailed functional organization are known

Our results show that some tracts are exclusively related to either the hind limb or forelimb region of the cord Other tracts such as the dorsomedial tract activated from low threshold cutaneous afferents and terminating in the lateral cervical nucleus (*cf* LUNDBERG and OSCARSSON 1961), seem to originate from both hindlimb and forelimb levels Further studies are needed to determine whether the differences between hindlimb and forelimb tracts reflect various anatomical arrangements subserving the same functional task, as with the DSCT and the cuneocerebellar tract or if they sometimes reflect more fundamental differences in the organization of afferent channels from hindlimbs and forelimbs

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Catecholamine Excretion and Personality Traits in Paratroop Trainees

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Abstract

Bloom G U S v Euler and M Frankenhaeuser *Catecholamine excretion and personality traits in paratroop trainees* Acta physiol scand 1963 *58* 77—89 — The excretion of adrenaline and nor adrenaline in urine was measured in paratroop trainees and officers during night rest ground training and during 2—3 hour periods under which jumps were performed from training tower and from aircraft The mean adrenaline excretion in the trainees was 3 3 $\pm$ 0 5 ng/min during night rest and 8 8 $\pm$ 1 0 ng/min during ground activity For periods including jumps mean adrenaline excretion was significantly increased to values of 17 4—19 9 ng/min The mean noradrenaline excretion was 11 2 $\pm$ 2 0 ng/min during night rest 23 6 $\pm$ 2 6 ng/min during ground activity and 21 1—38 1 ng/min in periods under which jumps were performed No difference in catecholamine excretion values was observed between officers and trainees Increased pulse rates were noted during preparatory procedures preceding a jump and lowered blood eosinophil levels observed after the 1st parachute jump Within the present small sample of subjects it was not possible to obtain reliable coefficients of correlation between basic personality traits and excretion of adrenaline and noradrenaline but some of the rating techniques used for assessing personality traits proved highly reliable and hence well suited for future research in this area

The quantitative evaluation of reactions to stress has attracted increasing interest in recent years both from the point of view of obtaining information on the bodily changes occurring under stress situations and with regard to the possibility of selecting personnel suitable for special tasks and missions

In addition to measurement of circulatory parameters such as heart rate and blood pressure attempts have been made to utilize other functions which are known to be influenced by the autonomous nerve system such as galvanic skin resistance and EEG (SIMONS 1961) The relationships between the adrenocortical system and stress, first pointed out by SELYE (1950) has led to studies of the excretion of steroids and steroid metabolites in urine and steroid levels in blood Changes in the urinary output of 17 ketosteroids have been reported as a result of stressful activities (motor car races boat races test flying) (PINCUS and HOAGLAND 1943 FROST DRYER and KOHLSTAEDT 1951) but do not seem to be a constant feature in stress (CONNEL COOPER and REDFEARN 1958 LEVI 1961)

The development of the technique for the differential estimation of noradrenaline and adrenaline in urine has made it possible to study more in detail the effect of stress on the activity of the adrenergic nerve system on the one hand and the adrenomedullary secretion on the other In this way it has been demonstrated that following varying types of mental stress such as apprehension anxiety irritation and excitation an increased excretion of catecholamines ensued as a regular phenomenon On most occasions in which the situation chiefly had provoked apprehension and anxiety or otherwise was perceived as disagreeable an increased adrenaline output has been observed while the noradrenaline excretion has been, on the whole unchanged. Increased adrenaline excretion has been reported in connection with examinations (PEKKARINEN et al 1961) under stressful working conditions (LEVI 1961) during emotional stress (EULER et al 1959) and in association with psychological tests (FRANKENHAEUSER and POST 1962) In other situations usually involving excitation with or without physical activity, noradrenaline output has been increased (GOODALL and BERMAN 1960 ELMADJIAN HOPE and LAMSON 1957 SILVERMAN and COHEN 1960 FRANKENHAEUSER and KÅREBY 1962, FRANKENHAEUSER STERKY and JARPE 1962)

Results from some of these investigations indicate a differential catecholamine excretion in different emotional states anxious reactions tend to be associated with increased adrenaline excretion and aggressive reactions with increased noradrenaline excretion (with or without increased adrenaline) The findings suggest that catecholamine excretion might be related to basic personality traits In this connection it may be recalled that physical work alone causes an increased output of noradrenaline (EULER and HELLNER 1952) The effect of flying on the output of catecholamines in urine has previously been subject to a limited study (EULER and LUNDBERG 1954) showing that air transportation as such may increase the adrenaline output in unexperienced subjects Increased catecholamine excretion has also been reported during supersonic flight tests (HALE 1962

During a period of military service at the Paratroop Training Center of the Swedish Army located in Karlsborg Sweden favourable conditions were

offered for one of us (G B) to study the reactions toward the stress involved in the rather specific activity of parachute jumping The investigation has included a study of heart frequency rates in connection with training flights blood eosinophil levels before and after parachute jumps and also an estimation of catecholamine excretion in urine during night rest under ground activity and after jump from training tower as well as from aircraft in flight Furthermore, estimates of some personality traits were obtained by ratings performed by the trainees themselves as well as by officers in charge of the trainees

Material and methods

In this investigation 15 Swedish Army soldiers in airborne training were randomly chosen from a platoon of 40 recruits which had volunteered for the paratroop branch of the services The platoon itself had previously been selected from three original platoons i e around 120 men in all

During paratroop training a certain period is almost exclusively devoted to the parachute jumping itself This period generally of 2—3 weeks duration includes intensive ground training in landing techniques flight training in order to accustom the trainees to flying and to situations which may occur while airborne jumping from various training towers — providing the sensation of jumping into and falling through space as well as an opportunity for training landing techniques — and lastly toward the end of this period the trainees will perform several jumps from aircraft in flight

The group of trainees was studied from the beginning of the special jump training period to the eighth parachute jump For the sake of comparison and also to establish whether or not an alteration in mode of reaction may follow upon frequent parachute jumping a group of non commissioned and commissioned officers having performed from 14 to 80 jumps was included in the material

Heart rate

The heart frequency of the trainees was measured in connection with their first training flights These flights which do not involve any jump are included in the training program in order to familiarize the trainees with airborne conditions On these occasions the subjects are confronted with the sensation of standing in the open exit viewing the ground while flying at the exact altitude and with the same speed to be maintained in the first real jump Pulse rates were determined upon the first training flight a) in the aircraft while grounded immediately before take off and b) while airborne directly upon returning to seat in plane from position at open exit

Blood eosinophil levels

Eosinophil counts were made on blood smears upon three occasions during the investigation period Blood samples were taken from the finger tip of the subjects and smears were made immediately The latter were air-dried and fixed in methanol Differential counts were performed on the smears after staining with Giemsa Two hundred leukocytes were counted in every smear and the percentage of eosinophils calculated on the basis hereof The blood counts were performed at a hospital hematology laboratory by personnel well trained in such procedures

The first eosinophil count was made on trainees at the same time that night excretion values of catecholamines were determined i e before starting ground training for parachute jumping but after about 3 months of ordinary infantry drilling The

Table 1 Pulse rate catecholamine excretion and blood eosinophil levels in trainees

Subject	Training flight Pulse rate		Night rest		Ground activity		Tower training	
			A	NA	A	NA	A	NA
	Before	After	ng/min		ng/min		ng/min	
	stress exposure							
1	90	110	—	—	4 9	18 2	15 1	57.2
2	84	84	6 2	15 5	7 1	20 4	—	—
3	90	96	1 7	5 5	8 5	14 1	—	—
4	78	84	—	—	3 8	17 9	10 9	34 7
5	88	108	—	—	12 9	34 3	16 8	11 9
6	100	120	1 7	6 8	11 3	25 5	20 9	41 0
7	72	90	3 9	14 6	15 3	21 3	18 0	44 9
8	72	78	4 4	13 9	16 0	51 4	21 8	40 3
9	60	70	2 1	6 3	5 6	18 2	10 5	33 5
10	90	102	3 3	6 7	7 7	11 6	20 8	42 0
11	70	78	—	—	5 9	15 0	15 7	41.3
12	84	96	3 3	20 4	7 5	34 5	—	—
13	100	120	—	—	10 8	22 7	13 7	33 7
14	74	80	—	—	7 7	23 5	11 2	35 6
15	84	88	—	—	6 7	25 2	17 8	41 4
Mean and S E.M	82 4±2 9	93 6±4 0	3 3±0 5	11 2±2 0	8 8±1 0	23 6±2 6	16 1±1 1	38 1±3 0

second count was made in connection with the first jump in the training tower and the third count lastly was made on blood samples taken 1/2—1 hour after the first jump from aircraft

Estimation of adrenaline and noradrenaline excretion in urine

Urine voided voluntarily was collect d and adjusted to pH 3 ± 0 2 by addition of 1 N hydrochloric acid At the start and the end of the collection period the bladder was emptied and the volume measured Samples of about 50 ml were kept in the re frigerator and sent within two days to the laboratory for analysis Previous tests have shown that the catecholamine content of the urine is maintained unchanged at the pH used during several days at room temperature (Euler and Lishajko 1961)

Night urine was collected between 10 p m and 7 a. m Samples were also collected during 2—4 hours of ground duty including routine exercise and during periods of 2—3 hours including jumps from the training tower and from the aircraft

Urine samples were collected from each of 8 officers on two occasions a) under a 2—3 hour period of non strenuous routine activities at the camp and b) under a period of similar length in which a parachute jump was performed

The urine samples were either kept in the deep freeze in the laboratory until analyzed or immediately processed according to the technique of Euler and Lishajko (1961) The method involves adsorption on alumina after adjustment to pH 8 2 with 1 N NaOH elution with 0 25 N HAc oxidation with ferricyanide after adjustment of pH to 6 2, transformation to lutines with alkali and ascorbic acid and reading in a fluorimeter using 2 filter sets according to Cohen and Goldenberg (1957) This technique allows differential estimation of free adrenaline and noradrenaline Recovery of added catecholamines is 75—85 per cent All values are uncorrected and expressed as hydrochlorides of the amines in ng per min

First jump		Sixth jump		Eighth jump (night)		Eos 2/6 (night rest)	Eos 9/6 (tower training)	Eos. 13/6 (1st jump)
A	NA	A	NA	A	NA			
ng/min		ng/min		ng/min				
17.5	17.1	—	—	27.3	35.1	1	1	2
—	—	—	—	30.0	53.7	0	1	—
12.0	9.4	25.0	38.3	16.9	31.5	2	1	1
23.2	24.8	—	—	12.0	20.6	7	6	1
34.7	26.3	—	—	25.3	26.1	4	5	1
17.2	16.2	—	—	25.0	49.2	1	—	1
19.7	22.5	17.0	34.4	19.8	42.5	4	2	3
—	—	26.3	37.6	28.4	35.0	2	0	—
7.4	11.7	10.3	34.3	9.4	35.6	5	5	3
13.2	9.1	17.9	27.0	17.6	20.1	—	3	1
16.3	19.8	—	—	21.0	36.3	1	2	0
21.1	27.2	—	—	15.7	18.4	—	3	4
27.0	35.4	12.0	25.9	19.7	35.1	3	2	0
22.5	29.3	13.1	47.8	15.0	31.4	3	—	0
19.6	25.6	—	—	—	—	1	4	—
19.3±1.9	21.1±2.2	17.4±2.4	34.3±2.3	19.9±1.6	33.6±2.8	2.2±0.4	2.6±0.5	1.4±0.4

Ratings of personality traits

Each subject rated all subjects including himself in 7 personality variables. In addition 6 officers rated those subjects with whom they were well acquainted. Thus 3 sets of ratings were obtained: fellow ratings, officers' ratings and self ratings. The subjects were assured that the ratings would be treated confidentially. The procedure, instructions and definitions of variables followed closely those used by MAGNUSSON (1959).

Each variable was first characterized by a general definition. The ratings were made on a 7 point scale, the two endpoints of which were defined as illustrated in the following example for the variable "oppositional":

1 Looks up to and admires uncritically a person in authority

4 Average

7 Oppositional
Defiant
Will not submit to authority

The other variables were: extrapunitive, maladjusted, anxious, cheerful, sociable and vital.

The ratings were performed for one variable at a time for all individuals in the group. For each subject the mean of all fellow ratings and the mean of all officers' ratings (in most cases 3—4) were used for each variable.

Results

The results of the various phases of the investigation are presented in Tables I and II and represent values obtained from the recruits during different situations in their training and from officers before and after parachute jump. In the tables only those subjects have been included from which both

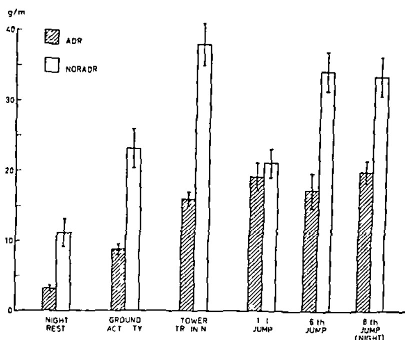


Fig 1 Adrenal ne and noradrenaline excretion in ng min in paratrooper trainees during n ght rest ground activity tower training and during 1 st 6th and 8th (night) jump Mean and S E M

values and values during one or the other form of jumping have been obtained The statistical significance of differences in heart rate blood eosinophil levels and catecholamine excretion during the various experimental condi tions has been determined by t tests of mean intra pair differences (see *e g* FISHER 1948 for a description of the application of the t test to differences in single individuals)

Heart rate

As seen in Table I heart frequency was increased in 14 of the 15 subjects and unchanged in one individual after experiencing the sensation of standing in the open exit of the plane viewing the ground while travelling at the same altitude and speed later to be used in actual jumps The mean increase was 11 2 beats per minute and the range 0—20 ($P < 0\ 001$)

Blood eosinophil levels

Eosinophil counts in the trainee group on blood samples taken in the be ginning of the jump training period while only ground exercise was being carried out showed an average percentage of 2 2 $\pm$ 0 4 A count made on samples collected after the first jumps in a training tower showed similar

Table II Catecholamine excretion in officers ng/min mean ± S E M

Subject	Number of jumps	Ground activity		Jump	
		A	NA	A	NA
1	44	13.1	33.8	24.9	44.5
2	16	6.0	20.2	24.0	38.2
3	60	7.2	16.5	17.4	63.7
4	14	14.5	32.2	24.7	35.7
5	31	6.6	14.1	9.9	26.3
6	16	6.8	21.1	9.1	21.7
7	80	8.8	22.6	14.6	24.7
8	60	11.5	22.6	24.2	33.2
Mean and S.E.M		9.3 ± 1.2	22.9 ± 2.4	18.6 ± 2.4	36.0 ± 4.8

values (2.6 ± 0.5) In blood samples taken shortly after the first jump from an aircraft in flight the eosinophil level was markedly lowered (1.4 ± 0.4) The difference between eosinophil levels during ground activity and the first jump was statistically significant ($P < 0.01$)

Catecholamine excretion in urine

As seen in Table I and in Fig 1 the night excretion figures are low both for adrenaline and for noradrenaline in the trainee group which is in agreement with earlier observations on resting healthy subjects

During ground activity involving a certain amount of physical exercise, the excretion rates are about twice as high as during night rest which again is in agreement with earlier experience (EULER HELLNER BJORKMAN and ORWEN 1955 KARKI 1956) The mean adrenaline values are less than 10 ng/min indicating a low activity of the adrenal medulla

Excretion values are also given from periods including tower training and jumps from aircraft on 3 different occasions the first the sixth and the eighth the last one being performed at night During these conditions the mean excretion of adrenaline was significantly higher than during ground activity at the same period of the day ($P < 0.001$ for the differences between ground activity and tower training first and eighth (night) jump $P < 0.05$ for the difference between ground activity and sixth jump) The adrenaline excretion was also significantly higher during the eighth (night) jump than during tower training ($P < 0.05$) whereas the excretion during the other conditions involving jumps either in the training tower or from the aircraft did not differ significantly The noradrenaline values were relatively high in three of the series but normal in one The excretion during the sixth and eighth jump was significantly higher than during ground activity ($P < 0.05$)

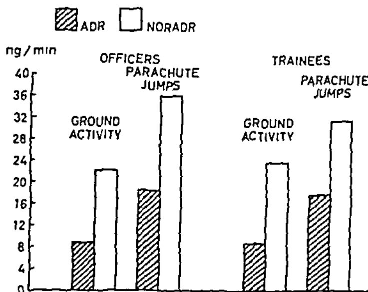


Fig 2 Average adrenaline and noradrenaline excretion in ng/min in officers and trainees during ground activity and parachute jumps

The catecholamine excretion values obtained from the officers group did not differ in any respect from those of the trainees as seen from Table II and Fig 2 In Fig 2 the average excretion figures are given for officers and trainees the values from all jumps in Table I being averaged

Personality traits as related to catecholamine excretion

Reliability of personality ratings — The reliability of the fellow ratings and the officers ratings was estimated by the split half technique (Spearman Brown s formula) In addition coefficients of correlation between (a) fellow ratings and officers ratings (b) self ratings and fellow ratings, and (c) self ratings and officers ratings were calculated The coefficients of correlation are shown in Table III

The coefficients of reliability obtained for the fellow ratings were high and the officers ratings although based on fewer data also showed a satisfactory reliability Likewise the agreement between fellow ratings and officers ratings was reasonably good except for the variable intropunitive — extrapunitive Self ratings on the whole showed higher correlations with fellow ratings than with officers ratings For both sets of data the variables intropunitive — extrapunitive and confident — anxious showed low correlations This indicates that self ratings may provide additional information in respect of personality traits that are not manifested in behaviour

Reliability of catecholamine values — Table IV shows the coefficients of correlation for adrenaline and noradrenaline, respectively under the various ex

Table III Reliability coefficients for fellow ratings and officers ratings (split half coeff) and coefficients of correlation between fellow ratings officers ratings and self ratings

Variable	Fellow ratings	Officers ratings	Fellow ratings and officers ratings	Self ratings and fellow ratings	Self ratings and officers ratings
1 Believing in authority—oppositional	93	.58	73	52	69
2 Intropunitive—extrapunitive	94	68	[illegible]	.29	− 04
3 Well adjusted—maladjusted	9[illegible]	68	51	64	63
4 Confident—anxious	84	36	67	.20	10
5 Gloomy—cheerful	88	90	63	67	36
6 Unsociable—sociable	92	78	80	66	54
7 Lacking vitality—vital	94	76	81	51	15

Table IV Coefficients of correlation for adrenaline and noradrenaline values from different experimental conditions

	Adrenaline			Noradrenaline		
	First jump	Sixth jump	Eighth jump (night)	First jump	Sixth jump	Eighth jump (night)
Tower training	− 03	.89	84	−.36	− 03	− 15
First jump		−.26	44		− 04	− 11
Sixth jump			69			.26

Table V Coefficients of correlation between ratings and means of catecholamine values from all stress conditions

Variable	Adrenaline			Noradrenaline		
	Fellow ratings	Officers ratings	Self ratings	Fellow ratings	Officers ratings	Self ratings
1 Oppositional	−.3[illegible]	−.36	− 3	40	.26	− 09
2 Extrapunitive	− 03	− 03	− 58	.25	32	− 10
3 Maladjusted	−.26	01	− 0[illegible]	2[illegible]	.57	39
4 Anxious	[illegible]5	18	6[illegible]	1[illegible]	35	63
5 Cheerful	−.28	−.21	05	− 07	− 45	− 1[illegible]
6 Sociable	−.25	− 07	15	− 13	− 18	−.2[illegible]
7 Vital	− 13	− 02	−.28	− 3[illegible]	−.35	− [illegible]

perimental conditions For adrenaline most correlations were reasonably high whereas for noradrenaline they were low and mostly negative

Correlations between personality traits and catecholamine excretion — The main comparison was based on the individual means of the catecholamine values for all stress conditions (tower training first, sixth and eighth jump) The correlations between these mean values and the various ratings are shown in Table V

The correlation coefficients were on the whole low compared with the standard error for a zero correlation, which for the present sample size is 0 267 The agreement between the two sets of correlation coefficients for fellow ratings and officers ratings was reasonably good whereas the self ratings showed a somewhat different pattern, in particular the disagreement was pronounced for the variables intropunitive — extrapunitive and confident — anxious

Discussion

Parachute jumping appears to provide a rather unique situation in which to study the reaction of an individual to the anxiety and stress of a tense life situation The voluntary jumping into space from a fast moving aircraft undoubtedly involves strong potential and even real threats to life and therefore lacks nothing in the way of tension The perhaps greatest advantage, at least from an investigatory point of view is the rarely encountered constancy and reproducibility of such a situation

The group of airborne volunteers studied here constitutes a rather homogenous group with regard to *e g* age, sex and physical health Although the group can thus hardly be considered as representative of *e g* the total population the homogeneity of the sample is in itself an experimental advantage since it limits some sources of variability although at the same time it limits the generality of the findings (BASOWITZ *et al* 1955)

The results presented indicate important effects in the vegetative sphere in subjects performing parachute jumps Previous investigations have shown that even transportation of untrained subjects by air involves a certain mental stress manifesting itself by increased release of adrenaline from the adrenal medulla and subsequent increased excretion through the urine (EULER and LUNDBERG 1954) It is therefore hardly surprising that parachute jumps, even in selected personnel should give rise to reactions conducive to increased adrenaline secretion It is also interesting to note that the average reaction was almost identical in trainees and in officers whose experience was greater This suggests that the reactions to the stress involved in parachute jumps are not subject to habituation such as has been shown to occur during repeated exposures to gravitational stress in a human centrifuge (FRANKENHAEUSER *et al* 1962)

The adrenaline values obtained in night urine and during ground activity were similar to those obtained in other studies The increase as a result of parachute jumping was relatively moderate but it should be noted that the figures represent the average excretion rate over 2—3 hours and that the actual peak secretion might have been considerably higher On the other hand it appears likely that the anticipation of the jump would have con tributed to the increased figures since anticipation stress has previously been shown to give rise to a marked increase in adrenaline output (FRANKENHAEUSER and KÅREBY 1962) The noradrenaline values were low during the night rest as shown previously and increased during ground activity in day time Part of this effect is due to the upright position (EULER LUFT and SUNDIN 1955) but it is probable that physical activity included in the training program has contributed to this effect Except for the first jump the nor adrenaline values were fairly uniformly increased

The specific nature of paratroop duties requires agility and stamina The importance of physical fitness is therefore specially emphasized in paratroop training and superb physical health is a main qualification for eligibility to this branch of military service It is conceivable that the moderate average increases in catecholamine excretion is a consequence of these qualities re flecting good mental balance in a stressful situation

A drop in the number of blood eosinophils has been reported in a wide variety of physical and psychological stresses by BASOWITZ *et al* (1955) The findings in the present investigation of a blood eosinophil response in connec tion with the trainees first parachute jump are in good agreement with their results These authors found also in paratroop trainees that while severe physical activity did not cause a drop in eosinophil numbers anticipation and jumping from a 250-foot mock tower or a plane in flight produced a marked eosinopenia

The present material is too small to allow a systematic investigation of the problem of a possible correlation between basic personality traits and catechol amine excretion during stress The data collected have been presented mainly to illustrate in a preliminary way the application of personality rating tech niques to psychophysiological problems

The high reliability coefficients obtained for the fellow ratings show that the technique was well suited for the conditions of the present experiment The coefficients of correlation for the catecholamine values from the various experimental conditions showed a large variability and are difficult to inter pret The relatively higher correlations for adrenaline as compared with nor adrenaline excretion may indicate that adrenaline excretion is a basic emer gency reaction of an organism exposed to stress whereas the noradrenaline response may be associated with complex psychophysiological relationships and hence the amount excreted may tend to vary for the same individual in different stress situations

The correlations that might exist between personality traits and catechol amine excretion will necessarily be weakened, or even obscured by the low reliability of the individual catecholamine values Hence the present data only serve to provide suggestions for further investigations In so far as the correlation coefficients (Table V) exhibit a consistent pattern they may be tentatively interpreted as indicating that oppositional extrapunitive and maladjusted individuals have high noradrenaline and low adrenaline levels whereas cheerful sociable and vital individuals have low levels and anxious individuals high levels of both catecholamines The negative correlations between noradrenaline excretion and vitality may indicate that vital (energetic active) individuals, when confronted with a stress situation, need not mobilize their emergency mechanisms as do individuals lacking vitality

This investigation was made possible by the kind cooperation of the staff of the Airborne Training Unit in Karlsborg as well as of the paratroop recruits who participated as subjects We are especially indebted to the commanding officer Major L Sjöström for his keen interest in the project The skilful assistance of Miss Karin Nicolausson and Mrs Inger Pettersson is gratefully acknowledged Mrs Paula Patkai has rendered valuable assistance in the computational work.

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Arterielle O_2Hb-Sättigung und alveolarer CO_2- und O_2-Druck der Ratte bei Aufenthalt in 2 000—7 000 m Hohe

von

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Abstract

Tribukait B *Arterielle O_2Hb Sattigung und alveolarer CO_2 und O_2 Druck der Ratte bei Aufenthalt in 2000—7000 m Hohe* Acta physiol scand 1963 58 90—98 — Rats were subjected to hypoxia corresponding to an altitude of 2 000—7 000 m The oxygen saturation in arterial blood of anesthetized animals was determined by the van Slyke method For determination of alveolar carbon dioxide and oxygen tensions un anesthetized animals were kept in a closed breathing system to which a small amount of carbon monoxide was added From the pressures of oxygen and carbon monoxide in this system and the COHb and O_2Hb saturations together with the alveolar gas equations the alveolar carbon dioxide and oxygen pressures were calculated The O_2Hb saturation decreased from 91 % at sea level to 83 65 56 and 39 % at 2 000 4 000 6 000 and 7 000 m altitude respectively The calculated carbon dioxide pressures decreased from a predicted value of 40 mm Hg at sea level to 35 32 17 and 14 mm Hg at 2 000 4 000 6 000 and 7 000 m altitude respectively These values for CO_2 tensions are in general agreement with those observed in man

Von den Untersuchungen des Menschen und der verschiedensten Tiere her ist wohlbekannt dass O_2 Mangel die Erythropoiese stimuliert Wo jedoch der Sauerstoff bzw Sauerstoffmangel in das komplexe Geschehen der Regulation der Erythropoiese eingreift ist unklar Als ein erster Schritt zur Losung dieses Problems ist die Erythropoiese der Ratte in Abhangigkeit vom Sauerstoffdruck der Einatmungsluft und zwar bei hohem O_2 Druck und Hypoxie untersucht worden Da relative Blutwerte — Erythrozyten oder Hb Konzentration —

nur einen begrenzten Einblick in die Grosse der Erythropoiese zulassen ist bei diesen Untersuchungen vor allem auch das Gesamt Hb gemessen worden (TRIBUKAIT 1962 a, b)

Zur weiteren Analyse dieser Befunde erschien es wunschenswert die Ver anderungen der Blutwerte nicht nur zum O_2 Druck der Einatmungsluft sondern auch zum O_2 Druck und zur O_2Hb Sattigung des arteriellen Blutes relatieren zu konnen Blutgasanalysen bei der Ratte unter Hypoxie scheinen jedoch zu fehlen

In der vorliegenden Arbeit sind deshalb derartige Bestimmungen durch gefuhrt worden — die arterielle Sauerstoffsattigung direkt nach VAN SLYKE der alveolare O_2 bzw CO_2 Druck nach einem indirekten Verfahren das eine Narkose uberflussig macht In einer nachfolgenden Arbeit sollen dann an Hand dieser Ergebnisse die Veranderungen der absoluten und relativen Blut werte bei variierendem O_2 Druck der Einatmungsluft analysiert und diskutiert werden (TRIBUKAIT 1962 c)

Material und Methodik

Die Untersuchungen wurden an etwa 300 g schweren mannlichen Ratten (Wistar) durchgefuhrt. Zur Bestimmung der arteriellen O_2Hb-Sattigung nach VAN SLYKE (VAN SLYKE und NEILL 1924) wurden die Tiere zunachst fur mindestens 3 Tage in kleinen Unterdruckkammern Hypoxie entsprechend 2000—7000 m Hohe ausgesetzt Die 7000 m Tiere waren zuvor 3 Wochen lang in 6000 m Innerhalb von 30 min wurden dann die Tiere in eine fur menschlichen Gebrauch geeignete grosse Unterdruck kammer[1] uberfuhrt und dort erneut vor der Blutentnahme wenigstens 30 min lang der entsprechenden Hohe ausgesetzt In Athernarkose wurde den Tieren von der Bauch aorta etwa 5 ml Blut anaerob mit einer silikonisierten und heparinisiert n Glasspritze entnommen Entsprechend den Angaben von BARTELS et al (1959) war zur Glycolyse und Autoxidationshemmung der verwendeten 5 % igen Heparinlosung eine 10 % ige NaF Lösung in gleicher Menge zugesetz Bis zur Analyse wurden die Proben in eisge kuhltem Wasser aufbewahrt

Die Hamoglobinkonzentration von Blutproben wurde als Oxyhamoglobin in 0 04 % iger Ammoniaklosung spectrophotometrisch (Beckman B) bei einer Wellenlange von λ = 545 mμ gemessen

Die indirekte Bestimmung des alveolaren O_2- bzw CO Drucks beruht auf dem Hb-Sattigungsverhalten von CO und O_2 bei Atmung von CO in einem geschlossenen Atem system Das Atemsystem sowie die Methoden zur Bestimmung von CO und O_2 in der Atemluft und von COHb bzw O_2Hb in Blutproben von 0 05—0 1 ml sind in einer fruheren Arbeit ausfuhrlicher beschrieben worden auf die verwiesen sei (TRIBUKAIT 1960)

Ergebnisse

Der arterielle O_2 Druck hangt vom alveolaren O_2 Druck und der alveo laren arteriellen O_2 Druckdifferenz ab der alveolare O_2 Druck vom O_2-Druck der Einatmungsluft und dem Grad der Ventilation im Verhältnis zum

[1] Herrn Dr v Dobeln der die Unterdruckkammer des Gymnastischen Centralinstitutes, Stockholm grosszug g zur Verfugung stellt b n ich zu besonderen Dank verpfl chtet

Tab I Luftdruck O_2 Druck der Einatmungsluft [F_{O_2} (P_B—47)] arterielle O_2Hb-Sättigung alveolarer CO_2 Druck ($P_{A_{CO}}$) daraus berechneter alveolarer O_2 Druck (P_{A_O}) der Ratte bei verschiedener Hohe Mittelwerte mit mittlerem Fehler

n = Anzahl der entsprechenden Versuche

Die Hohen Druckbeziehung entspricht DIN 5450

Hohe m	Luft druck mm Hg	O_2 Druck [Fo (P_B-47)] mm Hg	n	O_2Hb % Sättigung	n	$P_{A_{CO}}$ mm Hg	P_{A_O} mm Hg	Tage in Unter druck
0	760	149	22	90 6 ± 0 98				
2 000	596	115	8	83 1 ± 3 59	9	34 5 ± 2 05	73 6	14
					8	34 8 ± 0 96	73 2	28
4 000	462	87	8	64 9 ± 3 98	9	32 1 ± 1 13	48 4	11
5 000	405	75	7	59 2 ± 4 11	8	19 4 ± 3 77	51 6	3
6 000	354	64	7	56 3 ± 1 48	19	16 7 ± 1 61	44 2	2—3—4
					20	17 2 ± 1 51	43 6	39—99
7 000	308	55	7	39 4 ± 3 27	10	13 5 ± 3 70	38 4	3—4

Stoffwechsel d h dem CO_2 Druck und dem respiratorischen Quotienten (s Gleichung 2 alveolare Gasdruck Gleichung) Die arterielle O_2 Sättigung ergibt sich aus dem O_2 Druck und dem Charakteristikum der O_2Hb Dissoziations kurve Ausführlichere diesbezügliche Daten von hohenadaptierten Menschen unter verschiedenen Hypoxiebedingungen finden sich u a bei HURTADO und ASTE SALAZAR (1948) RAHN und OTIS (1948) s ferner Handbook of Respiration (1958)

Est ist nun aber nicht ohne weiteres moglich diese Resultate auf andere Sauger wie die Ratte zu ubertragen Deren hypoxische Atemreaktion ist praktisch unbekannt und die O_2Hb Dissoziationskurve weicht so wesentlich von der des Menschen ab (JONES et al 1950) dass mit Sicherheit fur einen gegebenen O_2 Druck wesentlich andere O_2Hb Sattigungswerte als beim Menschen zu erwarten sind

1 *Arterielle O_2Hb Sattigung unter Hypoxie*

Die arterielle O_2Hb-Sättigung ergibt sich aus dem O_2 Gehalt und der O_2 Kapazitat des Blutes Die O_2 Kapazitat wurde in einer Untersuchungsserie von Blutproben zunachst direkt bestimmt Sie betrug fur 12 Blutproben von Normaltieren 1 31 ± 0 003 ml O_2/g Hb und fur 11 Proben von Unterdrucktieren 1 28 ± 0 013 ml O_2/g Hb Die Differenz ist statistisch nicht signifikant (p = 0 10—0 05) Aus diesen Konstanten und der Hamoglobinkonzentration wurde dann bei den aktuellen Messungen die O_2 Kapazität errechnet

Aus Tab I sind die arteriellen O_2Hb Sattigungswerte bei Meereshöhe 2 000 und 4 000—7 000 m Hohe zu ersehen Bei einer mittleren O_2Hb-Sätti

gung von 66 5 % betrug der von 38 Doppelproben bestimmte Variation koeffizient ± 2 2 % entsprechend ± 1 5 O_2Hb-Sattigungsprozenten

2 *Alveolarer O_2 und CO_2 Druck unter Hypoxie*

Aus praktischen Grunden war es nicht moglich direkt im Blut den CO_2 bzw O_2 Druck zu messen Es erschien jedoch ein indirektes Verfahren gang bar, das vorteilhafterweise eine Narkose mit einer eventuellen Einwirkung auf die Atmung uberflüssig macht

Setzt man Tiere in einem geschlossenen Atemsystem CO aus so gilt nach dem sich ein Gleichgewicht eingestellt hat fur das Verhaltnis der alveolaren O_2 und CO Partialdrucke (P_{AO_2} P_{ACO}) und die O_2 bzw COHb-Sattigung (SO_2Hb SCOHb) die Haldane sche Gleichung

$$M = \frac{P_{AO_2}\ SCOHb}{P_{ACO}\ SO_2Hb} \quad (1)$$

Ist M bekannt und bestimmt man P_{ACO} SCOHb und SO Hb erhalt man P_{AO_2} Fur P_{AO_2} gilt weiter entsprechend der alveolaren Gasdruckgleichung (BENZINGER 1950)

$$P_{AO_2} = (P_B - 47)\ F_{O_2} - P_{ACO_2}\left[\frac{1}{RQ} - F_{O_2}\left(\frac{1}{RQ} - 1\right)\right] \quad (2)$$

P_B ist der aktuelle Barometerdruck 47 mm Hg betragt der Wasserdampfdruck bei 37 C F_O ist die Fraktion (Konzentration) von O_2 in der Einatmungsluft RQ der respiratorische Quotient Sind P_{AO_2} und RQ bekannt kann die Glei chung nach P_{ACO_2} aufgelost werden

In den folgenden Versuchen wurde entsprechend dieser Überlegung von Tieren die verschieden lange Zeit an Hypoxie adaptiert waren zum Versuch aber akut in Meereshohe gebracht worden waren der P_{AO_2} bestimmt Voraus setzung dafur, dass die so erhaltenen P_{AO_2} bzw P_{ACO_2} Werte auch fur den Hohenaufenthalt selbst Gultigkeit haben ist dass sich die Atmung wahrend der Versuchszeit von etwa 2 Stunden in Meereshohe gegenuber Hohenaufent halt noch nicht verandert hat Es ist ferner praktisch wichtig dass sich die CO Affinitat des Blutes von Hohentieren nicht von derjenigen normaler Tiere unterscheidet Eine unveranderte CO Affinitat ist namlich Voraussetzung dafur das ein gleicher M Wert unter allen Bedingungen in Gleichung (1) eingesetzt werden kann Um diese Frage zu prufen wurden 2 ml Blut von Tieren die sich 4—6 Tage in Hypoxie entsprechend 5 000 m befanden 4—36 Stunden mit einem stromenden Gemisch von 0 0199 % CO in Luft bei 37 C und 100 %iger H_2O Sattigung in Tonometern nach LAUÉ (1951) aquilibriert Die Tonometer waren gegen Licht geschutzt Mit jeder hypoxischen Blutprobe wurde gleichzeitig eine Normalblutprobe welcher Plasma bis zu einer gleichen

Tab II COHb-Sättigung von Blutproben hypoxischer (A) und normaler Ratten (B) äquilibriert mit 0 0119 % CO in Luft bei 37 C und Lichtabschluss für 4—36 Stunden

Versuch Nr	SCOHb %		Diff.
	A	B	
1	4 7	6 6	+ 1 9
2	6 9	6 4	− 0 5
3	5 9	4 9	− 1 0
4	6 5	6.3	− 0.2
5	8 0	9 0	+ 1 0
6	10.2	10 7	+ 0.5

Hämoglobinkonzentration abgenommen worden war, in gleicher Weise behandelt Aus Tab II geht hervor, dass keine systematische Differenzen in der COHb Sättigung zwischen Normalblut und Hypoxieblut bestehen Es ist ferner von Interesse dass nach 36 stündigem Tonometrieren (Versuch Nr 6) — eine Zeit, in der sicher ein volles Gleichgewicht zwischen den Gasdrucken und den Sättigungsgraden erreicht wird — die beiden errechneten M Werte denselben Nennwert (201, 210) haben wie der von Normaltieren *in vivo* bestimmte (204 s u)

Damit ist die Voraussetzung gegeben von hypoxieadaptierten Tieren aus Gleichung (1) direkt P_{AO_2} zu berechnen M bestimmt von 24 Normaltieren unter Berucksichtigung alveolarer O_2 Druckverhaltnisse (P_{ACO_2} = 40 mm Hg Blood Elliott und d Amour 1946, RQ = 0 80 Griffith und Farris 1942) betrug im Mittel 203 7

Entsprechend Gleichung (2) wurde ferner P_{ACO_2} berechnet wobei ein RQ von 0 80 (Sundstroem und Michaels 1942) angenommen wurde Aus diesen bei Meereshohe gewonnenen P_{ACO_2} Werten und den O_2 Drucken der Einatmungsluft unter Hypoxie ergeben sich wiederum unter Anwendung der alveolaren Gasdruckgleichung die alveolaren O_2-Drucke der entsprechenden Hohen

Aus Tab I sind neben dem O_2 Druck der Einatmungsluft die so erhaltenen alveolaren CO_2 und O_2 Drucke bei Hohenaufenthalt in 2 000 und 4 000—7 000 m wahrend verschieden langer Zeit ersichtlich

Diskussion

Die gefundenen alveolaren CO_2 Werte lassen erkennen dass zwischen dem O_2 Druck der Einatmungsluft und dem alveolaren CO_2 Druck keine lineare Beziehung besteht Bis zu etwa 4 000 m Hohe sinkt der CO_2 Druck relativ langsam nach Unterschreiten von 4000 m jedoch rascher ab Dieses Verhalten

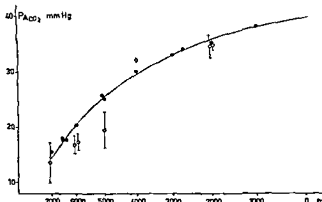


Abb 1 Alveolarer bzw arterieller CO Druck beim Hohenaufenthalt von Mensch (geschlossene Symbole mit mittlerem Verlauf) und Ratte (offene Symbole mit mittlerem Fehler der Mittel werte) Die Daten der hohenadaptierten Menschen sind folgenden Arbeiten entnommen DILL et al (1937) LUFT (1941) RAHN und OTIS (1948) HURTADO und ASTE-SALAZAR (1948) RILEY et al (1951) Die Werte der Ratte in 2 000—5 000 m Hohe repräsentieren je 8—9 Tiere in 6 000 m Hohe 19 bzw 20 Tiere nach 2 bis 4 und 39 bis 99-tägigem Aufenthalt und in 7 000 m Hohe 10 Tiere

entspricht im Ganzen gesehen den beim Menschen gemachten Erfahrungen Das geht aus Abb 1 hervor wo die Daten hohenakklimatisierter Menschen mit den eigenen Resultaten verglichen werden

Auch mit einem prinzipiell anderen indirekten Verfahren bei der Ratte gelangt man zu sehr ahnlichen Werten In einem subcutan oder intraperitoneal angelegtem Gasdepot (N_2) stellen sich nach einiger Zeit CO_2 und O_2 Drucke ein die weitgehend den venosen Gasdrucken dieser Zirkulationsgebiete entsprechen und in einer gewissen Relation zu den arteriellen Gasdrucken stehen (RAHN und CANFIELD 1955) VERZÁR und VOGTLI (1958) fanden so bei einer grosseren Zahl von Ratten in 1 880 m Hohe einen CO_2 Druck von rund 33 mm Hg in 3 450 m Hohe einen CO_2 Druck von etwa 28 mm Hg und in 6 000 m Hohe einen CO_2 Druck von rund 17 mm Hg subcutan bzw intra peritoneal Auch mit dieser Methode kann man also unter gewissen Annahmen zu einer Auffassung des arteriellen bzw alveolaren CO_2-Drucks gelangen ohne dass die Atmung der Tiere durch Eingriffe wie eine Narkose verandert wird Dies dürfte einer der wesentlichen Vorteile des oben beschriebenen eigenen Verfahrens sein Ein Nachteil dabei ist dass Annahmen gemacht wer den normaler $P_{ACO_2} = 40$ mm Hg RQ = 0 8 die im Einzelfall nicht völlig zutreffen mogen

Der Nutzeffekt eines derartigen Abfalls des CO_2 Drucks mit sinkendem O_2 Druck ist ohne weiteres klar um praktisch den gleichen Betrag um den der CO_2 Druck fällt steigt der O_2 Druck. Dieses Verhalten kann zu der eigen artigen und vom atmungsregulatorischen Standpunkt aus gesehen interessanten

Tab II COHb-Sattigung von Blutproben hypoxischer (A) und normaler Ratten (B) aquilibriert mit 0 0119 % CO in Luft bei 37 C und Lichtabschluss fur 4—36 Stunden

Versuch Nr	SCOHb %		Diff
	A	B	
1	4 7	6 6	+ 1 9
2	6 9	6 4	– 0 5
3	5 9	4 9	– 1 0
4	6 5	6 3	– 0 2
5	8 0	9 0	+ 1 0
6	10 2	10 7	+ 0 5

Hamoglobinkonzentration abgenommen worden war, in gleicher Weise behandelt Aus Tab II geht hervor, dass keine systematische Differenzen in der COHb Sattigung zwischen Normalblut und Hypoxieblut bestehen Es ist ferner von Interesse dass nach 36 stundigem Tonometrieren (Versuch Nr 6) — eine Zeit, in der sicher ein volles Gleichgewicht zwischen den Gasdrucken und den Sattigungsgraden erreicht wird — die beiden errechneten M Werte denselben Nennwert (201 210) haben wie der von Normaltieren *in vivo* bestimmte (204 s u)

Damit ist die Voraussetzung gegeben von hypoxieadaptierten Tieren aus Gleichung (1) direkt P_{AO} zu berechnen M, bestimmt von 24 Normaltieren unter Berucksichtigung alveolarer O_2 Druckverhaltnisse (P_{ACO_2} = 40 mm Hg Blood Elliott und d Amour 1946 RQ = 0 80 Griffith und Farris 1942) betrug im Mittel 203 7

Entsprechend Gleichung (2) wurde ferner P_{ACO_2} berechnet wobei ein RQ von 0 80 (Sundstroem und Michaels 1942) angenommen wurde Aus diesen bei Meereshohe gewonnenen P_{ACO_2} Werten und den O_2 Drucken der Einatmungsluft unter Hypoxie ergeben sich wiederum unter Anwendung der alveolaren Gasdruckgleichung die alveolaren O_2 Drucke der entsprechenden Hohen

Aus Tab I sind neben dem O_2 Druck der Einatmungsluft die so erhaltenen alveolaren CO_2 und O_2 Drucke bei Hohenaufenthalt in 2 000 und 4 000—7 000 m wahrend verschieden langer Zeit ersichtlich

Diskussion

Die gefundenen alveolaren CO_2 Werte lassen erkennen dass zwischen dem O_2 Druck der Einatmungsluft und dem alveolaren CO_2 Druck keine lineare Beziehung besteht Bis zu etwa 4 000 m Hohe sinkt der CO_2 Druck relativ langsam nach Unterschreiten von 4 000 m jedoch rascher ab Dieses Verhalten

nichtnarkotisierten Hund (ALBERS und USINGER 1956) und Kaninchen (WANG, WIRZ und VERZAR 1951) gefunden Im ganzen stimmen aber doch die erhaltenen O_2Hb Sattigungswerte relativ gut mit den erwarteten uberein wenn man von ahnlichen CO_2 druckbedingten Lageveranderungen der Dissoziationskurve wie beim Menschen ausgeht Schliesslich sei noch auf die relativ geringe Streuung der Werte hingewiesen, die auf eine ziemlich gleichmässige Atemreaktion der Tiere unter Hypoxie hinweist

In Abb 2 sind weiter die Dissoziationskurven des Menschen und zweier in Hohe lebender Tiere, des Llama und des Vicuna eingezeichnet worden Am Beispiel der O_2Hb Dissoziationskurven dieser Hohentiere ist zu erkennen, wie wesentlich es zu sein scheint und welche Wege die Natur geht einen hohen arteriellen O_2 Gehalt unter Hypoxie zu erreichen Die hohe arterielle O_2Hb Sattigung als Folge dieser linksverlagerten Dissoziationskurve ist jedoch nur dann von Vorteil wenn gleichzeitig auch der O_2 Gewebsdruck so niedrig ist dass O_2 tatsachlich vom Hamoglobin freigesetzt werden kann Aus dem Vergleich der Dissoziationskurven geht weiter hervor wie extrem ungeeignet die Ratte von diesem Gesichtspunkt her betrachtet fur einen Hohenaufenthalt sein muss

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Evidence of Adrenergic Neurons with Synaptic Terminals in the Retina of Rats Demonstrated with Fluorescence and Electron Microscopy

By

TORBJORN MALMFORS

Reserpinized animals exhibit a pronounced sensitivity to light turn away from moderate light and close their eyes At the same time they show an extreme miosis Biochemically it has been shown that reserpine causes a depletion of the monoamine depots in the amine-containing nerve tissues (*cf* CARLSSON *et al* 1957) which then cease to function The cause of this increased sensitivity to light might thus be that an aminergic inhibitory mechanism has got out of order To prove this it is necessary to demonstrate the presence of aminergic neurons in the visual pathway To show such cells the method of FALCK *et al* (1962) for direct localization of certain monoamines on the cellular level has primarily been applied to the retina of albino rats

For fluorescence microscopy halved rat eyes have been freeze-dried and treated with formaldehyde gas embedded in paraffin and sectioned in the usual manner according to the method described by FALCK (1962) In the retina of untreated rats a well-defined zone of fine yellow green fluorescent fibres appears between the inner plexiform layer and the inner nuclear layer The fibres are accumulated around non fluorescent cell bodies and have the characteristic appearance of terminal nerve fibres (*cf* HILLARP 1959 FALCK 1962) with typical varicosities which fluoresce intensely Noradrenaline in the hypothalamus has been shown to be concentrated in synaptic terminals of this appearance (CARLSSON FALCK and HILLARP 1962) In the same layer of the retina a small number of fluorescent cell bodies can be found from which fluorescent fibres can be seen to arise The specific yellow green fluorescence appears neither in the fibres nor in the cell bodies in specimens from animals treated with 10 mg reserpine/kg body weight 24 hours before preparation or with m tyrosine administered i p in 3 doses of 400 mg kg each with 2 hours

interval, the last injection 2 hours before the animals were killed There is thus good reason to believe (*cf* CARLSSON *et al* 1962) that the fluorescent product is derived from a monoamine, probably a catecholamine which is a primary amine (dopamine or noradrenaline)

As the fluorescent fibres remain intact 48 hours after opticotomy and bilateral excision of the cervical sympathetic chain of which the latter causes a complete disappearance of the amine fluorescence in the adrenergic nerves of iris there are reasons to suppose that this is a question of intraretinal adrenergic neurons provided with intraretinal synaptic terminals

The retina has also been examined with electron microscopy to get additional evidence of the presence of aminergic neurons For electron microscopy the retina has been fixed by means of intraocular injections of 40 per cent osmic acid in carbon tetrachloride, dehydrated in alcohol, embedded in epon sectioned in the usual manner and stained with lead tartrate In the layer of the specific fluorescence in the normal retina there are to be found nucleated vesicles in nerve cell processes and in a few nerve cell bodies These vesicles have the same appearance as those which have been found in adrenergic nerves (LEVER and ESTERHUIZEN 1961, ZELANDER, EKHOLM and EDLUND 1962 RICHARDSON 1962) and which seem to be identical with the amine containing granules (*cf* VON EULER 1958)

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Der Einfluss von Cobalt auf die Erythropoiese der Ratte

von

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Eingegangen am 16. Oktober 1962

Abstract

TRIBUKAIT, B. *Der Einfluss von Cobalt auf die Erythropoiese der Ratte.* Acta physiol. scand. 1963. *58.* 101—110. — The development of polycythemia in rats treated with cobalt was studied. Cobalt was injected intraperitoneally 6 times a week in increasing doses of 0.4, 0.6 and 0.8 mg/100 g body weight during 21, 22 and 45 days respectively. The total amount of hemoglobin, hemoglobin concentration, hematocrite, reticulocytes and blood volume were determined. Hemin tagged *in vivo* with C^{14}-2-glycine was isolated from 0.1—0.2 ml blood samples and the specific activity was measured. The hemoglobin concentration, hematocrite and reticulocytes increased significantly during treatment with 0.4 mg cobalt, but the total hemoglobin was unchanged. There were no signs of hemolysis as evaluated from the specific hemin activity. During treatment with 0.6 and 0.8 mg cobalt the relative blood values increased further and also the total hemoglobin increased. The hemoglobin concentration reached values of 20 g/100 ml of blood and the total hemoglobin increased from 2 to 3 g. The blood volume was unchanged. The reticulocytes reached a maximum value after about 40 days treatment and thereafter decreased to normal concentrations despite the increasing total hemoglobin. The degree of this polycythemia was similar to that one developed at an altitude of about 4000 m.

Die regulatorischen Prozesse der Erythropoiese zielen im allgemeinen darauf ab, den arteriellen O_2-Gehalt konstant zu halten. Ist der arterielle O_2-Gehalt herabgesetzt wie bei Anämie, Hypoxie, Kohlenmonoxyd-Vergiftung oder Methämoglobinämie, so steigt die Erythropoiese. Diese wird umgekehrt durch hohen arteriellen O_2-Gehalt, wie er z. B. bei Readaptation eines hohenpolyzythämischen Organismus an normalen O_2-Druck auftritt, gehemmt.

Dieses Regulationsziel der Erythropoiese wird unter dem Einfluss von Cobalt nicht mehr beibehalten. WALTNER und WALTNER (1929) beschrieben bei der Ratte nach Gabe von Co erhohte Hamoglobinkonzentrationen und Erythrozytenwerte. Diese Befunde sind wiederholt bestatigt worden (Literatur s. SCHULTZE 1940, GRANT und ROOT 1952). Untersuchungen des Blut- bzw. Erythrozytenvolumens haben daruber hinaus den absoluten Charakter der Polyzythämie gezeigt (ORTEN et al. 1933, DAVIS 1940, STANLEY, HOPPS und HELLBAUM 1946, GOODMAN 1947, BERLIN 1951, FISHER 1959).

Der zugrunde liegende Mechanismus dieser Stimulation ist unklar. U.a. ist ein die Atmung junger erythrozytarer Elemente hemmender Effekt angenommen worden, wobei diese Zellen beschleunigt in die Zirkulation ubertreten sollen (BARRON und BARRON 1937). Eine direkte Stimulation durch Hypoxie des Knochenmarks infolge Vasoconstriction (DAVIS 1940) oder Vasodilatation (ORTEN und ORTEN 1945) wird jetzt allgemein abgelehnt. Untersuchungen *in vitro* uber den hemmenden Einfluss von Co auf Enzyme, die mit der Gewebsatmung im Zusammenhang stehen (LEVY, LEVISON und SCHADE 1950), beruhren unmittelbar das Problem einer histotoxischen Hypoxie des Gewebes als Ursache der erhohten Erythropoiese. Lebhaft wird die Frage von Ubertragersubstanzen (Erythropoietinen), die unter Co-Wirkung freigesetzt werden sollen, diskutiert (GOLDWASSER et al. 1957, 1958, JACOBSON et al. 1959 a, b, BROWN und MEINEKE 1958).

Die vorliegende Arbeit, die im Rahmen von Untersuchungen der Erythropoiese unter verschiedenen Bedingungen vorgenommen wurde (TRIBUKAIT 1962 a—e), befasst sich mit der Entwicklung der Cobalt-Polyzythamie.

Material und Methodik

Die Versuche wurden an mannlichen Ratten eines Stammes (Hooded rats, National Institute for Medical Research, Mill Hill, London) mit einem Ausgangsgewicht von etwa 200 g ausgefuhrt. Die Nahrung bestand aus einem speziellen Rattenbrot (Zusammensetzung s. TRIBUKAIT 1960 a), Hafer, Mohrruben und Wasser *ad libitum*.

Den Tieren wurde Cobalt als $CoCl_2 \cdot 6H_2O$ in einer Dosierung von 0,4, 0,6 und 0,8 mg/100 g Korpergewicht i.p. 6 mal wochentlich verabfolgt. Diese Menge war in 0,1 ml einer 0,9%igen NaCl-Losung enthalten. Die Kontrolltiere erhielten ein gleiches Volumen 0,9 %iger NaCl-Losung.

Die Gesamthamoglobinmenge wurde mit einer modifizierten alveolaren CO-Methode, die beliebig oft wiederholte Bestimmungen am selben Tier zulasst, gemessen (TRIBUKAIT 1960 b). Hamoglobinkonzentration und Hamatokrit, aus denen sich zusammen mit dem Gesamthamoglobin das Blut-, Plasma- und Erythrozytenvolumen berechnen, wurden vom Schwanzblut der Tiere in der fruher angegebenen Weise bestimmt (TRIBUKAIT 1960 b). Die Reticulozyten wurden von 2 mal 1000 Erythrozyten entsprechend der Methode von LARSSON und SWENSSON (1949) ausgezahlt. Dabei werden dunne Blutausstriche in 1 %iger Sublimatlosung fixiert und mit Toluidin-Losung (0,75 %, pH 5,7) gefarbt.

Eine allgemeine Information uber eventuelle hamolytische Prozesse lasst sich aus dem Verhalten radioaktiv *in vivo* gezeichneten Hamoglobins gewinnen. Wie zu Bestimmungen der Lebenszeit von Erythrozyten (FORSSBERG und TRIBUKAIT 1962 a) wurden etwa

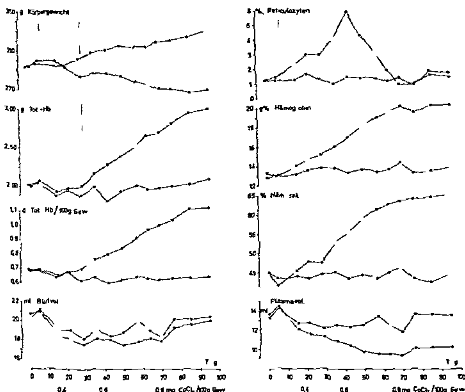


Abb. 1. Gewicht, Gesamthämoglobin, Gesamthämoglobin/100 g Körpergewicht, Blutvolumen und relative Blutwerte von Cobalt-behandelten Ratten (geschlossene Symbole) und Kontrolltieren (offene Symbole). Mittelwerte von je 10 Tieren

10 μC (0,169 mg) C^{14}-2-Glycin/Tier i. p. injiziert. Von dieser Aktivität finden sich beim normalen Tier 0,2—0,3% im Häminanteil des Hämoglobin in relativ konstanter Konzentration bis zum etwa 40. Tag nach der Injektion, wonach mit dem Ende der Erythrozytenlebenszeit auch die spezifische Häminaktivität absinkt. Ein vorzeitiger oder beschleunigter Abfall beruht entweder auf einer Hämolyse oder einer Verdünnung der spezifischen Aktivität durch einen Überschuss neugebildeten Hämins. Letzterer lässt sich aus der Grösse des Gesamthämoglobin erkennen. Um Komplikationen zu umgehen, die sich aus einem eventuell unterschiedlichen Verhalten der spezifischen Aktivität des Globin und Häminanteils des Hämoglobin ergeben können, war Hämin von 0,1—0,2 ml Schwanzblut isoliert worden, dessen spezifische Aktivität gemessen wurde. Für methodologische Einzelheiten s. FORSSBERG und TRIBUKAIT (1962 b).

Ergebnisse

Eine Übersicht über die wesentlichen Veränderungen der absoluten und relativen Blutwerte sowie des Körpergewichts bei successiv gesteigerter Co-Medikation vermittelt Abb. 1. Aus Tab. I sind ferner die Ausgangs- und Endwerte ersichtlich.

Tab 1 Gewicht und Blutwerte von je 10 Ratten nach 82 tägiger Behandlung mit CoCl bzw NaCl
I = Kontrollen II = Cobalt Tiere a = Ausgangswerte (Mittel von Bestimmungen an 2 Tagen)

	Gewicht g	Tot Hb g	g Tot Hb / 100 g Gew	Rel Hb g%	Hct %	Rel Hb g% / Hct % · 100
I a	295± 4 7	2 04±0 044	0 69±0 009	13 17±0 174	44 6±0 75	29 6±0.28
I b	331±10 5	2 11±0 064	0 64±0 009	13 80±0 167	44 8±0 49	30 8±0 30
II a	297± 6 9	2 04±0 035	0 68±0 010	13 01±0 126	43 4±0 40	30 0±0 17
II b	270±11 0	3 02±0 132	1 12±0 032	20 22±0 274	65 1±0 87	31 0±0 16

Der erste Versuchsabschnitt umfasst 21 Tage wobei 18 mal 0 4 mg *CoCl*/100 g Körpergewicht bzw NaCl Lösung injiziert wurden Das Körpergewicht der Cobalttiere fällt im Gegensatz zu dem der Kontrollen etwas Dieser Abfall kann auf einen leicht toxischen Effekt von $CoCl_2$ hinweisen Die Gesamt Hb Menge beider Versuchsgruppen sinkt initial um 5—7 % Die Differenz gegenüber den Ausgangswerten ist für die Kontrollen hochsignifikant ($p < 0,001$) nicht aber für die Cobalttiere Im weiteren Verlauf ist die Hb Menge beider Versuchsgruppen praktisch unverändert Wird das Gesamt Hb auf das Körpergewicht bezogen (g Tot Hb/100 g Körpergewicht), ergibt sich für die Kontrollen eine fallende für die Cobalttiere eine steigende Tendenz Die Hb-Konzentration *der Kontrollen ist praktisch unverändert die der Cobalttiere* steigt von 13 g % hochsignifikant ($p < 0\ 001$) auf 15 g % Der Hämatokrit folgt den Änderungen der Hb Konzentration während der ganzen Versuchszeit Damit ist die mittlere Hb Konzentration der Erythrozyten, ausgedrückt durch das Verhältnis von Hb Konzentration/Hämatokrit, unverändert Das Blutvolumen fällt bei beiden Versuchsgruppen hochsignifikant (*$p < 0\ 001$*) *um* etwa 10 % bei den Cobalttieren etwas stärker als den Kontrollen Dieser Abfall beruht teils auf einem Absinken des Plasmavolumens das für die Cobalttiere hochsignifikant ($p < 0\ 001$) für die Kontrollen signifikant ($p = 0\ 01$—$0\ 001$) ist teils auf den etwas niedrigeren Tot Hb Werten Die Reticulozyten der Cobalttiere liegen um das 2—3 fache hochsignifikant ($p < 0\ 001$) über den Ausgangswerten

Die Ergebnisse des ersten Versuchsabschnitts lassen sich dahingehend zusammenfassen das die relativen Blutwerte zwar deutlich unter Co Medikation ansteigen Das unveränderte Gesamt Hb zeigt jedoch dass es sich hierbei lediglich um Veränderungen im Sinne einer Hämokonzentration handelt *Ob* die erhöhten Reticulozytenwerte tatsächlich eine gesteigerte Erythropoiese anzeigen muss so lange offen bleiben als eine Hämolyse nachgewiesen werden kann Darauf wird weiter unten eingegangen

Während des *22 Tage dauernden zweiten Versuchsabschnitts* wurde die Cobaltdosis auf 0 6 mg/100 g Körpergewicht gesteigert 19 Injektionen wurden verabfolgt Mit einem 20 %igen Anstieg des Gesamt Hb um etwa 0 4 g und einer Steigerung der Hb Konzentration auf 18 g % hat sich jetzt eine echte

b = Endwerte. Mittelwerte mit mittlerem Fehler

Blut vol ml	ml Blut vol / 100 g Gew	Ery vol ml	ml Ery vol / 100 g Gew	Plasmavol ml	ml Plasmavol./ 100 g Gew	Ret ‰
20 7±0 36	7 0±0 07	6 9±0 16	2 3±0 03	13 8±0 28	4 7±0 07	12 8±1 11
20 4±0 63	6 2±0 11	6 9±0 25	2 1±0 03	13 6±0 40	4 1±0 08	13 8±1 76
20 9±0 37	7 0±0 13	6 8±0 12	2 3±0 04	14 1±0 27	4 8±0 11	13 6±1 14
20 0±1 05	7 4±0 18	9 7±0 43	3 6±0 10	10 3±0 59	3 9±0 10	16 8±1 95

Polyzythämie entwickelt. Entsprechend dem steigendem Erythrozytenvolumen das dem Gesamt-Hb parallel läuft und auf dessen Wiedergabe deshalb verzichtet wurde, ist das Plasmavolumen um beinahe 50 % gesunken, das Blutvolumen ist unverändert. Die Reticulozyten erreichen jetzt einen Maximalwert um 6 ‰ — das 5—6 fache des Ausgangswertes. Das Körpergewicht hält sich unverändert.

Im letzten Versuchsabschnitt wurden während 45 Tage 40 mal eine Dosis von 0 8 mg $CoCl_2$/100 g Körpergewicht gegeben. Hier fällt das Körpergewicht wiederum ab und liegt schliesslich 30 g unter dem Ausgangsgewicht und 60 g unter dem der Kontrollen. Das Gesamt-Hb steigt weiter und erreicht 3 g. Der Hb-Zuwachs nimmt gegen Ende des Versuchsabschnitts etwas ab. Rechnet man aber mit einer normalen Lebenszeit der während des vergangenen Versuchsabschnitts gebildeten Erythrozyten, so ist die Hb-Bildungsleistung etwa unverändert, da zu diesem Zeitpunkt in grösserem Umfang Erythrozyten abgebaut und ersetzt werden. Die auf das Körpergewicht bezogene Hb-Menge erreicht mit 1 12 g/100 g Gewicht beinahe das Doppelte des Normalwertes. Die Hb-Konzentration und der Hämatokrit scheinen sich einem oberen Grenzwert zu nähern. Da das Gesamt-Hb weiter steigt, ergibt sich daraus ein langsam ansteigendes Blutvolumen, das schliesslich gegenüber dem Ausgangswert vor Cobaltgabe unverändert ist. Das Plasmavolumen verbleibt während dieses Versuchsabschnitts auf einem unverändert niedrigen Niveau. Bemerkenswert ist das Verhalten der Reticulozyten, die auf normale Konzentrationen abfallen und nicht mehr von den Kontrollwerten abweichen.

Das Verhalten der spezifischen Aktivität von *in vivo* mit C^{14} 2 Glycin gezeichnetem Hämin ist aus Abb. 2a zu ersehen. Unter der Annahme eines Molekulargewichts von 68 000 für Hämoglobin und 650 für Hämin wurde weiter aus den jeweiligen Werten des Gesamt-Hb die Gesamthäminmenge berechnet, aus der sich zusammen mit der spezifischen Aktivität die gesamte Häminaktivität ergibt. Diese zeigt Abb. 2b.

In guter Übereinstimmung mit früheren Untersuchungen zur Bestimmung der Erythrozytenlebenszeit (FORSSBERG und TRIBUKAIT 1962 a) beginnt etwa um den 40. Tag nach der Injektion die Aktivität abzusinken und erreicht am 75. Tag bei beiden Gruppen etwa 13 % des Wertes vom 37. bzw. 38. Tag. Die

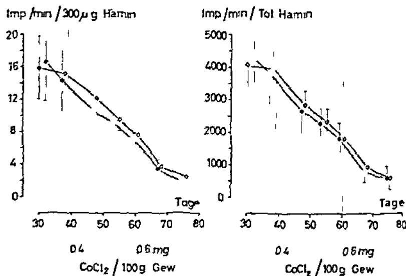


Abb. 2a, b. Verhalten der spezifischen und gesamten Aktivität von *in vivo* mit C^{14}-2-Glycin radioaktiv gezeichnetem Hämin. Mittelwerte mit Standardabweichung von 10 mit Cobalt behandelten Tieren (geschlossene Symbole) und 10 Kontrolltieren (offene Symbole). Die Zeitskala (Abszissenachse) bezieht sich auf den Tag der Injektion von C^{14}-2-Glycin (Tag 0)

Aktivität der Co-Tiere liegt am 38. Tag etwas unter derjenigen der Kontrollen. Unabhängig von der Co-Therapie zeigen dann die Kurvenverläufe der spezifischen wie der gesamten Aktivität eine weitgehende Parallelität zueinander. Es ist also weder eine Hämolyse nachweisbar, die einen stärkeren Abfall der spezifischen wie der gesamten Aktivität zur Folge haben sollte, noch ein stärkerer Zuschuss von neugebildetem Hämin während dieser Versuchsperiode zu erkennen, der zu einem stärkeren Absinken der spezifischen Aktivität bei unveränderter Gesamtaktivität führen sollte.

Diskussion

Die vorliegenden Untersuchungen bestätigen den mehrfach beschriebenen Befund einer echten Polyzythämie nach Zufuhr von Cobalt. Ein Vergleich der gefundenen Werte mit denen der Literatur ist vor allem wegen der unterschiedlichen Dosierung und Versuchszeit nur schwer möglich.

Das im wesentlichen unveränderte Blutvolumen bei einem Anstieg des Erythrozytenvolumens um beinahe das Doppelte und einem ensprechenden Abfall des Plasmavolumens stimmt gut mit den Befunden BERLINs (1951) überein, der das Erythrozytenvolumen mit P^{32}-gezeichneten Erythrozyten bestimmte. ORTEN et al. (1933) sowie STANLEY et al. (1946) haben mit Congorot bzw. Evans blue wesentlich höhere Ziffern gefunden.

Die Hb-Konzentration mit einem Endwert von 20 g % liegt etwas unter den von anderen Autoren für Ratten angegebenen Werten (ORTEN 1936, STANLEY

et al 1946 CRAFTS 1952) Allerdings waren auch deren Ausgangswerte hoher

Im allgemeinen werden standig oder vorubergehend erhohte Reticulozyten werte beschrieben (Literatur s GRANT und ROOT 1952) allerdings hat BERLIN (1951) normale Konzentrationen bei einem ebenfalls unveraendertem Differentialbild des Knochenmarks gefunden Die eigenen Resultate zeigen vorubergehend gesteigerte Reticulozytenkonzentrationen die aber nicht oder nur teilweise zum Anstieg des Gesamt Hb zugeordnet sind Aus den Reticulozyten alleine sind also nur mit Vorsicht Ruckschlusse auf die hamatopoietische Aktivitat eines Organismus unter Co Therapie moglich

Das um 50 % erhohte Gesamt Hb mit einem im wesentlichen unveranderten Blutvolumen entspricht dem Bild einer Polyzythamie wie sie bei einem langeren Aufenthalt in etwa 4000 m Hohe zu finden ist (TRIBUKAIT 1962a) Die mittlere Erythrozyten Hb Konzentration weicht ebenfalls nicht wie auch bei der Hohenpolyzythamie von der Norm ab Weiterhin scheint die Erythrozytenlebenszeit bei der Co Polyzythamie (VAN DYKE et al 1955) ebenfalls wie bei der Hohenpolyzythamie (FRYERS und BERLIN 1952) normal zu sein Auch der Myoglobingehalt bei der Co-Polyzythamie ist wie bei der Hohenpolyzythamie unverandert (FAULKNER und BLOOD 1957) Es finden sich also hier keine Anhaltspunkt dafur dass die Co Polyzythamie von der Hohenpolyzythamie abweicht

Die Entwicklung der Co Polyzythamie zeigt einige bemerkenswerte Einzelheiten Auf das Verhalten der Reticulozyten ist schon oben hingewiesen worden Die Hb Konzentration bzw der Hamatokrit steigen zunachst bei unverandertem Gesamt Hb Es liegen somit Zeichen einer Hämokonzentration mit einer Reticulozytose vor Letztere wird soweit aus dem Verhalten des radioaktiven Hamin hervorgeht — ein allerdings ziemlich grobes Verfahren — nicht durch eine Hamolyse ausgelost Eine Hamokonzentration wird ebenfalls initial nach einsetzender Hypoxie beobachtet (TRIBUKAIT 1962 b) Diese geht allerdings sehr rasch in eine echte Polyzythamie uber Im Gegensatz dazu entwickelt sich die Co-Polyzythamie wesentlich langsamer Das mag vor allem mit den Konzentrations und Ausscheidungsverhaltnissen von Co im Tier zusammenhangen

Im allgemeinen ist in den verschiedenen Untersuchungen Co peroral oder parenteral als Chlorid Sulphat Glutamat oder Nitrat in einer Dosierung zwischen etwa 0 1—1 0 mg/100 g Korpergewicht verabfolgt worden Eine Dosierung von 0 4 mg $CoCl_2$/100 g Korpergewicht hatte in den vorliegenden Versuchen nur einen Effekt auf die peripheren Blutwerte die Reticulozyten und das Korpergewicht Daraus lasst sich eine klare Wirkung dieser Dosierung erkennen die jedoch nicht zu einer echten Polyzythamie fuhrt Es ist jedoch denkbar dass eine langere Gabe eine echte Polyzythamie hervorgerufen hatte SAIKKONEN (1959) fand mit einer Dosis dieser Grossenordnung bei der Ratte eine gewisse Hypertrophie der Nebennieren und geringere reaktive Verande

rungen der Milz als Ausdruck einer allgemeinen Stressreaktion, jedoch keine erhöhte Ausscheidung von Coproporphyrin, wie sie als Zeichen einer stärkeren toxischen Reaktion bei 4 fach höherer Co-Dosierung auftrat

Die Wirksamkeit von Co hängt generell davon ab ob die Zufuhr von Co die Ausscheidung übertrifft wobei nach STARE und ELVEHJEM (1933) so n JOSLAND und MCNAUGHT (1938) die Gesamtmenge Co in der Ratte 40—50 μg erreichen muss bis eine Polyzythämie nachweisbar wird Als Normalwert wird etwa 5 μg angegeben Die Ausscheidung erfolgt relativ rasch und annähernd exponentiell. Nach COPP und GREENBERG (1941) sind 10 Stunden nach der Injektion von Co etwa 70 % durch die Nieren ausgeschieden und nach 96 Stunden mehr als 95 % CARLBERGER (1961) fand eine Ausscheidung von etwa 50 % innerhalb von 3 Stunden nach der Injektion Bei täglicher Zufuhr fanden CUTHBERTSON, FREE und THORNTON (1950) nach 14 Tagen 5—7 % der gesamten zugeführten Menge im Organismus nach vielwöchentlicher Medikation werden von STARE und ELVEHJEM (1933) eine Gesamtmenge entsprechend etwa 15—20 % der täglich zugeführten Menge angegeben Die Cobalt Konzentrationen in Niere Pancreas Leber und Milz sind wesentlich höher als in den übrigen Organen (COPP und GREENBERG 1941 BERLIN 1950 CUTHBERTSON et al. 1950 CARLBERGER 1961)

Als auslösende Ursache der Co-Polyzythämie ist häufig eine Gewebsanoxie diskutiert worden Die Untersuchungen von BARRON und BARRON (1937) nach denen die Atmung junger erythrozytärer Elemente durch Co gehemmt werden soll, konnten von WARREN SCHUBMEHL und WOOD (1944) nicht bestätigt werden Bei *in vitro*-Versuchen an Knochenmark fanden LAFORET und THOMAS (1956) keine Atmungshemmung durch Co bei Konzentrationen bis zu 10^{-3} M, Konzentrationen über 10^{-3} M hemmten jedoch die Häminsynthese Es scheint damit eine direkte Stimulation des Knochenmarks durch Co ausgeschlossen zu sein ganz abgesehen davon dass es zweifelhaft ist, ob überhaupt so hohe Konzentrationen *in vivo* im Knochenmark erreicht werden Ähnliche Überlegungen gelten auch für die Untersuchungen von BURK et al (1946) und LEVY et al. (1950) die eine Hemmung der Atmungs- bzw Enzymaktivität von Gewebsschnitten und Gewebshomogenaten bei einer Co-Konzentration von 10^{-3} M gefunden hatten Andererseits weist der Abfall des O_2-Verbrauchs der Ratte nach Co-Injektion (WESLEY 1956) auf deutliche Stoffwechselstörungen hin.

JACOBSON et al (1959 a b) haben eine Anoxie der Niere angenommen wodurch Übertragersubstanzen (Erythropoietine) freigesetzt werden sollen (GOLDWASSER et al 1957 1958) Plasma von Tieren nach einer einzelnen Co-Injektion hatte eine Wirkung gemessen am Auftreten Fe-gezeichneter Erythrozyten im Blut fastender Ratten die die Wirkung von Plasma anämischer Tiere nach einer massiven Blutung um das Vielfache übertraf Dieses Ergebnis ist und im Hinblick auf die langsame Entwicklung der Co-Polyzythämie bemerkenswert. BROWN und MEINEKE (1958) fanden in ähnlichen Versuchen einen wesentlich bescheideneren Effekt Eine endgültige Beurteilung dieser

Befunde durfte erst dann möglich sein wenn klar gelegt ist ob mit Plasma von Co-behandelten Spendertieren eine echte Polyzythamie hervorgerufen werden kann oder nicht

Es sei schliesslich noch erwahnt dass Co nicht die O_2 Kapazitat des arteriellen Blutes etwa durch Methamoglobinbildung herabzusetzen scheint (BUCCIERO und ORTEN 1949)

Zusammenfassend lasst sich feststellen dass die Co-Polyzythamie weit gehende Parallelen zur O_2 Mangelpolyzythamie aufweist Bei offenbar normalem arteriellen O_2 Gehalt ist jedoch die primare Ursache in Veranderungen auf dem Gewebsniveau zu suchen Ob beiden Polyzythamieformen ahnliche auslosende Mechanismen zugrunde liegen ist bislang unbekannt

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The Tissue Distribution of Radioactivity Following the Injection of Varying Levels of Fatty Acid Labeled Chylomicrons in the Rat

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Abstract

BELFRAGE P B BORGSTROM and T OLIVECRONA. *The tissue distribution of radioactivity following the injection of varying levels of fatty acid labeled chylomicrons in the rat* Acta physiol scand 1963 *58* 111—123 — H^3 palmitic acid labeled chylomicrons in doses of 4—124 mg lipid were injected intravenously into rigorously carbohydrate fed rats The radioactivities in blood liver adipose tissue spleen lungs, kidneys and muscles were studied For doses of 4 to 33 mg lipid the disappearance of label from the blood followed an exponential rate which was similar for these doses and corresponded to half lives of 2 2 to 3 5 minutes The maximal radioactivities found in the liver were also similar for these doses and ranged from 40 to 51 % of the injected radioactivity For doses of 64 and 124 mg lipid the disappearance curve was more complex and the label disappeared more slowly from the blood The maximal radioactivity found in the liver was also decreased It is concluded that at these doses the system was overloaded The spleen and the lungs took up significant amounts of chylomicron fatty acids as evidenced by initial peaks in the radioactivity curves for these tissues The kidneys the heart and the muscles showed a slower rise of radioactivity and presumably took up mainly recirculated fatty acids The adipose tissue also seemed to take up mainly recirculated fatty acids

It is well established that dietary fatty acids with a chain length of 14 carbons or more enter the circulation mainly as esterified fatty acids in chylomicron form (BORGSTROM 1960) The further fate of the chylomicrons have been partly elucidated during recent years (OLIVECRONA GEORGE and BORGSTROM 1961)

The triglycerides which make up the major part of the chylomicrons are removed quite rapidly from the circulation A considerable fraction can be found in the liver and experiments with chylomicrons, labeled both in the glycerol and in the fatty acid moiety of the glycerides have shown that the chylomicron triglycerides penetrate into the liver as such (BORGSTROM and JORDAN 1959 OLIVECRONA 1962 a) Chylomicron fatty acids can also be found in many extrahepatic tissues at short times after injection of fatty acid labeled chylomicrons (BRAGDON and GORDON 1958), but the mechanism by which they penetrate into these tissues is not known with any certainty

The quantitative role of the liver in the removal of chylomicron triglyceride from the circulation is not definitely established Studies of the tissue distribution of injected fatty acid labeled chylomicrons have given different results showing liver radioactivities corresponding to 15—50 % of the injected radioactivity (FRENCH and MORRIS 1958, BRAGDON and GORDON 1958 BORGSTROM and JORDAN 1959 OLIVECRONA 1962 a b) The physiological significance of of these results was made doubtful when BORGSTROM and JORDAN (1959) pointed out that the lipid doses injected in these experiments were unphysiologically high Therefore the present experiments were carried out to study the possible influence of the size of the lipid dose injected on the tissue distribution of labeled chylomicron fatty acids in the rat

Materials and Methods

Preparation of the chylomicron suspensions 5 mg corresponding to 5 mC of 9 10 H³ palmitic acid (The Radiochemical Centre Amersham England) was dissolved in a minute volume of ethanol neutralized with dilute KOH and diluted with water This solution was administered intragastrically to a rat on which a thoracic duct cannulation had been performed the previous day The rat was then given small volumes of an artificial milk preparation containing 3 % corn oil (Lactovit AB Mazetti Malmö Sweden) The chyle was collected for 12 hours in a flask chilled in ice Simultaneously unlabeled chyle was obtained from other rats given the same artificial dry milk preparation The chyle was concentrated by centrifugation for 30 min at 78 000 × *g* in a Spinco model L ultracentrifuge using the 40 rotor The top layer was withdrawn layered under 1 1 % NaCl and recentrifuged for 10 min under the same conditions The top layer was aspirated and resuspended in 1 1 NaCl Aliquots of the labeled chylomicrons were mixed with unlabeled chylomicrons to give the desired concentrations of lipid The smallest dose injected represented essentially undiluted labeled chylomicrons and contained 4 42 10^{8} CPM as measured under our counting conditions All other doses used contained the same amount of labeled chylomicrons plus increasing amounts of unlabeled chylomicrons The lipid content of the doses were 4 1 mg (CA) 5 7 mg (CB) 8 8 mg (CC) 13 mg (CD) 33 mg (CE) 64 mg (CF) and 124 mg (CG) The volumes were 1 0 ml except for the highest dose which had a volume of 1 5 ml Thus all rats in a given series were injected with the same amount of fat irrespective of their individual body weights

All injection and sampling procedures were completed within 20 days after the collection of the first chyle

Treatment of rats injection of chylomicrons and sampling Male Sprague Dawley rats weighing 210 ± 20 g (mean $\pm$ standard deviation of the samples) were used Th y were first fasted for 24 hours then allowed to drink 20 % glucose in half strength saline *ad libitum* for 12—20 hours Two hours before the injection of the chylomicrons they were given 5 ml of the same glucose solution by stomach tube

The chylomicrons were injected into an exposed jugular vein This was done under light ether anesthesia and the rats were allowed to wake up immediately after the injection At appropriate times they were again anesthetized with ether and blood drawn from the aorta Usually we obtained 7—9 ml of blood About 1 ml was immediately transferred to a stoppered centrifuge tube containing 10 ml of chloroform methanol 2 1 The exact amount of blood transferred was determined by weight The liver was removed rinsed in water blotted dry and immediately homogenized in chloroform methanol 2 1 The heart, both lungs the spleen both kidneys a piece of the abdominal wall muscles and a piece of an epididymal fat body were removed rinsed in water blotted dry and transferred to chloroform methanol 2 1 The tissues were transferred to the solvent within 3 min after the death of the animal and were homogenized at a later time

To measure the oxidation of the injected chylomicron fatty acids individual rats were killed immediately after the injection of chylomicrons and other rats at the longest times studied These rats were immediately homogenized in 1 l ethanol in a Warring blendor The homogenate was transferred to a 6 l Erlenmeyer flask diluted to 4 l with diethyl ether and allowed to stand at room temperature for at least 24 hours It was then filtered and an aliquot taken to dryness redissolved in chloroform methanol 2 1 and treated as the other tissue extracts The mean value for the rats killed at "zero time was taken as 100 % and the total recovery in the rats killed at later times calculated relative to this figure This value is given in the tables as total

Analysis performed on the samples The chloroform methanol 2 1 extracts of the tissues were filtered into separatory funnels and 0 4 volumes of aqueous 2 % KH_2PO_4 was added The funnels were shaken and after equilibration which usually took 12 hours the lower chloroform phase was transferred to an Erlenmeyer flask dried with anhydrous sodium sulphate taken to dryness and weighed The lipids were redissolved in chloroform and an aliquot pipetted into a scintillation counting vial The chloroform was evaporated off and the lipids dissolved in 10 ml of toluene containing 3 g PPO (2 5 diphenyloxazole) and 100 mg POPOP (1 4-bis 2 (phenyloxazolyl) benzene) per l and counted in a Packard liquid scintillation spectrometer model 314 AX All counts were corrected for quenching by use of internal standards

To the plasma lipid extracts in the stoppered centrifuge tubes was added 4 ml of 2 % KH_2PO_4 the tubes shaken and the phases separated by centrifugation The upper methanol water phase was aspirated and the lower chloroform phase filtered into liquid scintillation counting vials The lipids were then dissolved in toluene and counted as described above

The liver lipids from CA CE and CG were separated into glyceride and phospholipid fractions on silicic acid columns as previously described (OLIVECRONA 1962 c)

Results

The results obtained are shown in Table I and in Fig 1—4 All values are expressed as percent of injected radioactivity

Blood The blood radioactivities were obtained by measuring the lipid radioactivity in an aliquot of the sample withdrawn and multiplying with a factor for total blood content assuming this to be 8 g per 100 g body weight The

Table I Tissue distribution of label in refed male rats after intravenous injection of chylomicrons labeled with H^3 palmitic acid Values expressed as percent of injected radioactivity

Rat no.	Time (min)	Blood	Liver	Adipose tissue	Muscle	Heart	Kidneys	Spleen	Lungs	[illegible]
4 1 mg lipid										
CA 3	2	71 8	13.2	3 8	13 1	0 34	0 67	0 86	7 6	
CA 4	2	45 6	27 6	3 2	6 8	0 30	0 36	1 3	7.8	
CA 5	5	2 8	21 5	5 5	—	0 27	0 47	1 5	5 7	
CA 6	5	25.8	33 6	4 8	4 1	0 24	0 32	1 3	4 0	
CA 7	10	9.2	45 4	3 6	5 5	0.2[illegible]	0 42	0 6[illegible]	3 7	
CA 8	10	4.2	40.2	4 3	2 7	0.21	0 42	1 0	2 1	
CA 9	20	[illegible] 8	40 0	2 1	3 1	0 50	0 45	0.[illegible]	[illegible].2	
CA 10	20	2 8	37.2	1 8	13 4	0 [illegible]3	0 58	0 9[illegible]	2 7	
CA 11	80	2 [illegible]	19	8 0	7 5	0 31	0 72	0 50	1.5	
CA 12	80	1 8	[illegible]2 5	3 1	5 1	0.21	0 [illegible]3	0 40	1 7	
CA 13	80									8 0
CA 14	80									83 7
5 7 mg lipid										
CB 3	5	39 9	36 8	6 0						
CB 4	5	19 3	8 4	7 4						
CB 5	10	24 0	[illegible]6 0	5 7						
CB 6	10	15 4	37 5	3 1						
CB 7	15	[illegible]4	48 8	7 0						
CB 8	15	3.2	4[illegible] 5	2 5						
CB 9	30	2.2	34 5	3.8						
CB 10	30	[illegible] 2	33 6	4 1						
CB 11	80	2 1	[illegible] 8	10 5						
CB 12	80	2.2	[illegible]3 1	3 5						
8 8 mg lipid										
CC 3	5	[illegible]0 0	3[illegible]	2.8	3 3	0 19	0 4[illegible]	1 3	5 1	
CC 4	[illegible]	[illegible]3	40 [illegible]	[illegible]9	5 1	0 16	0 44	1 3	5 4	
CC 5	1	9 3	40.2	[illegible]6	3 4	0 18	0 40	2 1	3 3	
CC 6	1	[illegible] 0	41 6	3 1	2 9	0.24	0 4[illegible]	2.2	4.5	
CC 7	2[illegible]	3 3	33 8	[illegible]	3 4	0.22	0 49	1 [illegible]	[illegible] 7	
CC 8	2	3 4	3 [illegible]0	6[illegible]	3 4	0 19	0 45	1 4	3 9	
CC 9	40	2 [illegible]	30 [illegible]	13 [illegible]	5 [illegible]	0 34	0 61	0 8[illegible]	1.8	
CC 10	40	4 1	[illegible]0 4	[illegible]6	[illegible]0	0.28	0 43	1.2	2.3	
CC 11	80	2 [illegible]	[illegible]0	21 [illegible]	5.2	0 22	1.01	0 90	1.9	
CC 12	80	1 6		4 9	6 3	0 17	0 53	0.[illegible]9	[illegible].6	
CC 13	80									[illegible]6
CC 14	80									71 1

Table I (cont.)

Rat no.	Time (min)	Blood	Liver	Adipose tissue	Muscle	Heart	Kidneys	Spleen	Lungs	Total
13 mg lipid										
CD 3	10	9.9	44.3	5.0						
CD 4	10	10.2	50.8	4.9						
CD 5	20	3.5	49.9	4.3						
CD 6	20	3.[illegible]	41.3	4.2						
CD 7	30	2.4	44.0	3.4						
CD 8	30	1.8	37.8	[illegible].4						
CD 9	60	2.0	31.3	3.4						
CD 10	60	1.6	3[illegible].1	4.3						
CD 11	160	0.9	15.9	8.1						
CD 12	160	1.6	[illegible]1.1	9.2						
33 mg lipid										
CF 3	10	19.8	40.4	9.7	[illegible].7	0.15	0.48	3.3	4.1	
CF 4	1[illegible]	10.[illegible]	47.5	5.9	2.2	0.20	0.40	1.3	5	
CF 5	25	2.8	44.5	7.2	3.5	0.24	0.53	2.0	4.6	
CF 6	25	2.5	47.[illegible]	7.4	2.8	0.23	0.49	2.7	5.5	
CF 7	40	3.2	41.0	3.4	3.0	0.23	0.50	2.1	4.2	
CF 8	40	2.2	40.8	2.0	[illegible].5	0.23		2.1	4.1	
CF 9	80	2.8	[illegible]8.0	16.5	4.9	0.18	—	0.86	3.1	
CF 10	80	2.0	28.8	12.9	5.3	0.24	0.5[illegible]	0.90	2.[illegible]	
CF 11	160	1.6	20.3	[illegible]4.7	5.9	0.17	0.48	0.83	2.4	
CF 12	160	1.9	24.3	2[illegible].0	4.8	0.16	0.49	0.73	1.8	
CF 13	160									84.7
CF 14	160									93
64 mg lipid										
CF 3	15	47.2	23.5	[illegible].9						
CF 4	15	40.3	[illegible]1.5	6.[illegible]						
CF 5	30	[illegible]1.0	[illegible]4.5	9.[illegible]						
CF 6	30	21.9	6.8	30						
CF 7	45	3.4	24.1	[illegible]0.7						
CF 8	45	4.3	25.6	12.3						
CF 9	80	3.0	26.8	14.6						
CF 10	80	1.5	4.1	5.2						
CF 11	160	1.8	21.4	8.3						
CF 12	160	1.4	19.2	[illegible].8						

Table I (cont)

Rat no	Time (min)	Blood	Liver	Adipose tissue	Muscle	Heart	Kidneys	Spleen	Lungs	Total
124 mg lipid										
CG 3	20	52 6	23 8	4.5	4 2	0.25	0 63	3 7	3 8	
CG 4	20	36 2	[illegible]2 1	4 1	3 7	—	0 5[illegible]	4 1	2 8	
CG 5	40	11.5	34 1	11 8	4 6	0.29	0.54	1 9	3 6	
CG 6	40	2 4	36 6	2 2	4 1	0 18	0 42	3 1	2 7	
CG 7	60	3 7	27 3	9 7	—	0 32	0 57	1 3	2 [illegible]	
CG 8	60	2 4	32 0	4 3	4 3	0 21	0 58	2 0	2 0	
CG 9	80	1 9	22 7	5 5	5 7	0 15	0 68	0 66	1 6	
CG 10	80	2 0	27 4	7 4	4 4	—	0 63	1 6	1 8	
CG 11	160	0 84	17.3	10 6	4 9	0 17	0 67	0 32	1 6	
CG 12	*160*	*1 8*	*23 0*	*19 5*	*8 5*	*0.22*	*0.50*	*0 86*	*1 4*	
CG 13	160									8[illegible] 8
CG 14	160									[illegible]9 9

values obtained are plotted semilogarithmically in Fig 1 The label disappeared at approximately the same rate for the five low doses (4 1—33 mg lipid) and the first parts of these curves seemed to follow a single exponential Due to the limited number of rats studied and the variation between individual rats it is not possible to define the curves accurately (This restriction applies to all tissues studied) For the two high doses (64 and 124 mg lipid) the curves were more complex than for the low doses and the label also disappeared more slowly

Liver The liver radioactivities were calculated from the radioactivities in the aliquots of liver fat studied The figures were not corrected for radioactivity present in the blood remaining in the tissue BRAGDON and GORDON (personal communication) have measured the amount of blood present in tissues of rats after partial exsanguination using iodine labeled albumin and under these conditions found as a mean 0 094 ml plasma present per g of liver corresponding to around 10 % of the total blood We have generally obtained 7 to 9 ml of blood from our rats and the residual blood therefore should be less than calculated by BRAGDON and GORDON The contribution of radioactivity from the blood was significant only during the first time period when appreciable amounts of radioactivity were still present in the blood

For the five low doses (4 1—33 mg lipid) the maximal values for liver radioactivity were similar The range was 40—51 % of the injected radioactivity The rats injected with the two high doses (64 and 124 mg lipid) showed lower maximal values

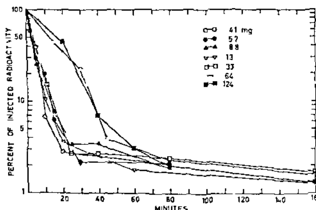


Fig 1 Disappearance of H palmitic acid labeled chylomicrons injected intravenously in rigorously carbohydrate fed rats Values expressed as percent of injected radioactivity

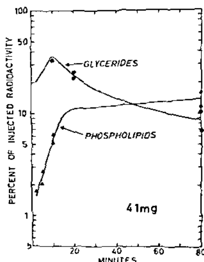


Fig 2 Radioactivities in liver glycerides and phospholipids in rats after injection of H palmitic acid labeled chylomicrons Dose injected 4 1 mg lipid per rat Values expressed as percent of injected radioactivity

Fig 2 3 and 4 show the radioactivities in the glyceride and phospholipid fractions respectively for three of the doses Initially most of the label was present in the glyceride fraction This fraction reached its maximal radioactivity early and this then declined simultaneously with a rise of the phospholipid radioactivity so that at the end of the experimental time more label was present in the phospholipid fraction than in the glyceride fraction

Other Tissues The radioactivity in heart lungs spleen and kidneys were calculated from the radioactivity found in aliquots of lipid extracts from these tissues The radioactivity in muscles was calculated from the radioactivity

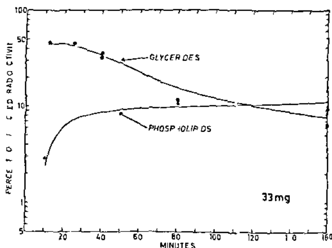


Fig 3 Radioactivities in liver glycerides and phospholipids in rats after injection of H^3 palmitic acid labeled chylomicrons Dose injected 33 mg lipid Values expressed as percent of injected radioactivity

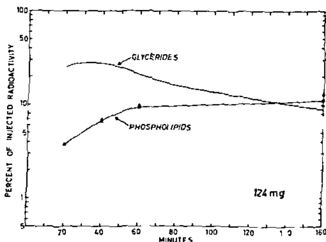


Fig 4 Radioactivities in liver glycerides and phospholipids in rats after injection of H palmitic acid labeled chylomicrons Dose injected 124 mg lipid. Values expressed as percent of injected radioactivity

the sample collected and multiplied by a factor for total muscle This factor was taken to be 45 % of the body weight This calculation involves the unproven assumption that all muscles had the same specific radioactivities (CPM/g wet weight) as the sample of abdominal wall muscle studied Therefore the values for muscle radioactivities are less reliable than those for liver, heart kidneys spleen and lungs

From the figures of BRAGDON and GORDON (personal communication) for the blood content of partially exsanguinated rats and our own figures for the weight of the organs it can be calculated that the heart would contain about 0 4 % the kidneys about 1 8 % and the muscles about 10 % of the blood The radioactivities found in these tissues at early times were so low that radioactivity in the blood remaining in the tissues could account for a major part Thus the radioactivity curves for these tissues would give a slow rise and then level off at plateaus which would be similar for the different fat doses At long times about 0 2—0 3 % of the injected radioactivity was found in the heart about 0 4—0 7 % in the kidneys and about 3—7 % in the muscles

The lung would contain about 1 3 % and the spleen about 0 7 % of the blood The radioactivities found in these tissues at short times are considerably higher than can be accounted for by their content of residual blood radioactivity Thus these tissues showed an initial rapid rise of radioactivity The radioactivity then declined rather rapidly Thus both the first and the second part of the radioactivity curve were different from that for heart kidney or muscle and resembled more that of the liver For the spleen the height of the maximum increased with increasing fat do es In contrast, the height of the maximum in the lung decreased with increasing fat doses

The values for adipose tissue radioactivity were obtained by multiplying the radioactivities in the samples actually collected by a factor for total adipose tissue Since, in this experiment the total lipid contents of the individual rats were not determined a mean figure was used This was derived from the lipid contents of the 16 rats used for determination of total The mean figure for these rats was 6 3 ± 1 0 g lipid per 100 g body weight (mean ± standard deviation of the samples) It was assumed that 70 % of these lipids represented adipose tissue lipids and that all adipose tissue lipids had the same specific activity as those actually measured The adipose tissue would contain about 4 % of the blood When this is taken into consideration the data generally does not show an initial peak of adipose tissue radioactivity but a slow gradual rise However the variation between individual rats and between the doses are fairly wide The data are less reliable than those for liver heart kidneys spleen or lungs since only a sample (epididymal adipose tissue) and not the entire adipose tissue mass was studied

Sum of radioactivity in the tissues studied At the shortest times the radioactivities in the tissues studied add up to 70 % or more of the dose injected At the longest times this sum is as low as 35 % in some cases The decline can be partly explained by oxidation of the fatty acids The results from the experiments where total activity was determined showed however that in all cases more than 70 % of the injected radioactivity could be recovered at the longest times This means that a considerable fraction of the radioactivity must have been located in tissues not sampled or that the samples were not representative for the tissues In recent identically performed experiments with labeled fat emul-

sions similar results were obtained In these experiments we therefore hydrolyzed the remains of the rats and measured the radioactivity in a petroleum ether extract of the acidified hydrolysate The radioactivity recovered was adequate to explain the discrepancy between the sum of blood, liver heart, muscle adipose tissue kidney, spleen and lung radioactivities and the radioactivity recovered in the homogenized whole rats Thus the methods used were adequate In comparison with the results obtained by BRAGDON and GORDON (1958) our values for adipose tissue and muscle are low

Discussion

The main purpose of the present work was to study the effect of the lipid dose on the tissue distribution of fatty acid labeled chylomicrons in rats

In earlier work on the tissue distribution of injected labeled chylomicrons in the rat, doses of lipid have been used, which initially give unphysiologically high concentrations of chylomicron lipid in the blood BRAGDON and GORDON (1958) injected 18 and 44 mg lipid in two experiments, FRENCH and MORRIS (1958) 40—60 mg BORGSTROM and JORDAN (1959) 21 5 mg and OLIVECRONA (1962 b) 120 mg all during the course of about 1 minute The normal influx of chylomicron lipid from the intestine in the rat has been estimated to 60—80 mg per hour (ABERDEEN SHEPHERD and SIMMONDS 1960) In the present study the amount of chylomicron lipid injected over 1 minute ranged from 4 1 to 124 mg per rat or approximately 20 to 600 mg per kg Calculated as rate of influx our lowest dose thus still exceeded the physiological range However after distribution over the blood volume the concentration of chylomicron lipid resulting from the lowest dose probably fell within the physiological range

) *Disappearance of chylomicron fatty acids from the circulating blood* FRENCH and MORRIS (1957) reported that when fatty acid labeled chylomicrons were injected intravenously into rats the major part of the label disappeared at an exponential rate They stated that the rate was inversely proportional to the amount of lipid injected The disappearance curves leveled off at a low level of radioactivity OLIVECRONA GEORGE and BORGSTROM (1961) showed that this break in the curve is mainly due to phospholipid fatty acids in the injected chylomicrons which disappear at a slower rate than the glyceride fatty acids Part of the break is also due to recirculation of label

CARLSON and HALLBERG (1963) have recently studied the elimination of intravenously injected fat emulsions in fasting dogs They found that below a concentration of 1 1 mM glyceride per liter blood the elimination was exponential i e a constant fraction was removed per time unit Above 1 1 mM per liter blood the elimination was linear i e a constant amount was removed per time unit The maximal removing capacity was calculated to be about 0 05 mM glyceride per liter blood and minute In a 200 g rat as used in this study this would mean about 0 7 mg triglyceride per minute per rat.

The present data are not sufficient to define the initial part of the disappearance curves accurately The data do not indicate any significant difference in the initial fractional disappearance rates for doses from 4 1 to 33 mg lipid per rat (Fig 1) With the two high doses 64 and 124 mg lipid per rat, the label disappeared more slowly from the blood and the disappearance curves were more complex than for the low doses (Fig 1) It seems that for the high doses the fractional disappearance rates were less in the first time interval than in the second These results do not agree with those of FRENCH and MORRIS but might be fitted to a pattern similar to that reported by CARLSON and HALLBERG In our experiment with the highest dose 124 mg lipid about 44 % of the label was left in the circulation at 20 min Thus about 70 mg lipid had left the circulation during these 20 min corresponding to at least 3 5 mg per min This would be a minimum figure for the maximal disappearance rate The figure is about 5 times higher than that calculated from the value for dogs

The second slow part of the blood radioactivity curve was quite similar for all doses The mean values for blood radioactivity were between 1 8 and 2 4 % of the injected dose at 80 min and between 1 3 and 1 7 % at 160 min At these fairly long times most of the label probably represented recirculation from the liver or other tissues From this limited evidence it thus seems that the fractional rates of these recirculations were independent of the amount of lipid injected Most probably this means that the labeled fatty acids at these times were mixed into fairly large pools

Liver Previous studies have indicated that the liver plays an important and perhaps unique role in the metabolism of chylomicrons There is evidence that chylomicron triglycerides are taken up intact by the liver (BORGSTROM and JORDAN 1959 OLIVECRONA 1962 a) The fraction of chylomicron fatty acids taken up by the liver is not definitely established Published figures are 20 % (BRAGDON and GORDON 1958) 25 % (FRENCH and MORRIS 1958) 33 % (BORGSTROM and JORDAN 1959) 40 % (OLIVECRONA 1962 b) and 35 % (OLIVECRONA 1962 a) All these figures represent fatty acid radioactivity after injection of labeled chylomicrons but have been calculated in different ways Thus BRAGDON and GORDON reported % of cleared activity found in the liver the next three figures are % of injected dose found in the liver and the last figure is the ratio of fatty acid radioactivity in the liver to total fatty acid radioactivity recovered None of these figures measure directly the fraction of chylomicron glyceride taken up by the liver We know that once the chylomicron triglyceride is taken up into the liver it is rapidly metabolized A fraction of the fatty acids is oxidized other fractions are reesterified into triglycerides or phospholipids A fraction of these newly formed esters are incorporated into lipoproteins and transferred to the plasma from where a fraction probably disappears into extrahepatic tissues On the other hand chylomicron fatty acids may also be retransported from extrahepatic tissues presumably as free fatty acids and a fraction taken up by the liver Thus the maximal radioactivity found in the liver is actually

the sum of primary uptake of chylomicron fatty acids oxidation and recircula tion and does not measure directly the fraction of chylomicron fatty acids taken up by the liver

The rats in the present experiment were rigorously carbohydrate fed to minimize the oxidation of the fatty acids and thus reduce one of the sources of error in the evaluation of the fraction taken up by the liver Still appreciable amounts were oxidized 15—30 % of the total fatty acid radioactivity were lost during the experimental time (80—160 min) This fixes the decay rate of the total system, or at least a mean decay rate during the experimental time The rate of decline of the liver radioactivity after the maximum had been reached was faster than the decay of the rest of the system This means that either the fractional rate of oxidation was higher in the liver than in the rest of the system or there was a net transport of label from the liver to other parts of the system In most cases both alternatives were probably true Thus in most cases the dif ference in radioactivity in the liver between the point of maximum and the last point measured was slightly larger than the difference between the total recovery at zero time and the last time This means that even if all oxidation took place in the liver and during the time interval between the maximum and the last point some net transfer of label from the liver to other tissues must have taken place Thus the maximal radioactivity in the liver is really a minimum figure for the primary uptake of chylomicron fatty acids by the liver After the uptake of a considerable fraction more than 50 % of the *chylo*micron fatty acids into the liver part of these are transferred from the liver to extrahepatic tissues

With doses of 4 1 to 33 mg lipid per rat about 50 % of the injected radioactivity could be found in the liver at short times For these low doses there was no appreciable variation of this figure with the dose At the two high doses however *the maximal liver radioactivity was appreciably* less and it appears likely that at these doses the liver became relatively saturated and its fractional rate of removal of chylomicrons from the circulation decreased

The decrease in the fractional disappearance rate of the label that occurred at 64 mg lipid cannot be accounted for only by a decrease in the fractional rate of removal of chylomicrons by the liver but must mean that the fractional rate of removal of chylomicrons by the extrahepatic tissues was also decreased It seems that at doses of 64 mg lipid or more there was a general overloading of the system the behaviour of which changed accordingly

After the uptake of chylomicron triglyceride into the liver a transfer of labeled fatty acids to the phospholipid fraction occurs (BORGSTRÖM and JORDAN 1959, OLIVECRONA 1962 a b) Whether this transfer proceeds via hydrolysis and re esterification of the liberated fatty acids or via a transesterification mechanism is not known Fig 2 3 and 4 show the glyceride and phospholipid radioactivities for 3 of the doses The transfer of label to the phospholipids occurred slower with higher fat doses This may indicate a slower utilization of the tri

glycerides in the higher doses or it may simply be a result of the lower specific activity of the fatty acids in the higher doses

Other tissues A primary uptake of chylomicrons by a tissue would give an initial rapid rise of radioactivity as was found for the liver Depending upon the rate of disposal of the fatty acids by the tissue, the radioactivity would then level off or decline The spleen and the lungs showed this type of curves with initial peaks of radioactivity and it may thus be concluded that these tissues take up significant amounts of chylomicron fatty acids which are then rapidly disposed of by the tissue Whether they are oxidized in the tissues or transferred back to the plasma and transported to other tissues cannot be determined by the present data

The heart the kidneys and the muscles on the other hand showed no initial peaks of radioactivity and when the contribution of radioactivity from residual blood in the tissues was taken into consideration they showed a slow rise of radioactivity as would be expected if they took up mainly recirculated label

The adipose tissue showed a small initial peak of radioactivity in some of the cases The dominant feature however was a secondary rise of radioactivity It seems probable that adipose tissue takes up mainly recirculated label and that it might be a major destination for the net transport of label from the liver

MRS IRÈNE GUDMUNDSSON MISS KERSTIN ROSENDAHL and MISS MARIETTE JOHNSSON gave skilful technical assistance

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From the Institute of Zoophysiology, University of Oslo, Norway

The Physiology of the Swimbladder of the Eel Anguilla vulgaris

I. The solubility of gases and the buffer capacity of the blood

By

JOHAN B. STEEN

Received 27 October 196[illegible]

Abstract

STEEN, J. B. *The physiology of the swimbladder of the eel Anguilla vulgaris. I. The solubility of gases and the buffer capacity of the blood.* Acta physiol. scand. 1963. 58. 124—137. — Some physiological properties of eel blood which may have importance in gas exchange in the swimbladder have been investigated. Using the Scholander and Roughton syringe analyzer for blood gases and micromethods for determination of lactic acid and pH the solubility of the gases of air has been measured at various temperatures and pH levels at pressures up to 1 atm. The buffer capacity of eel blood has also been studied in some detail. The O_2 and CO_2 solubility followed the same general pattern as is known to obtain in blood of other species. The O_2-content of blood at a pO_2 of 1 atm is reduced by about 40 % when the pH is lowered from arterial values to 7.0. The N_2 and Ar solubility is approximately 10 % reduced when the pH of eel blood is lowered from about 7.8 to 7.5. Using the same experimental technique no such effect was found on eel plasma, blood from man, trout or cod.

The volume of the teleostean swimbladder is regulated by secretion and reabsorption of the atmospheric gases. Gas reabsorption usually occurs in an anatomically specialized part of the bladder where gases can easily diffuse into the blood while gas secretion is accomplished by the gas gland. During secretion all these gases, with the possible exception of CO_2, are transported to the gland by the blood. Here gas is removed from the blood and deposited in the swimbladder at a higher partial pressure. In deep sea fishes the pO_2 inside the bladder may be several hundred times higher than in the water

All the other gases of air are also frequently found in the bladder at a higher pressure than they have in the water (SCHOLANDER and VAN DAM 1953) Numerous theories have been advanced to explain this ability of the swim bladder to concentrate gases (*Vide* HALL 1924 and DENTON 1961 for reviews) They differ mainly in the significance ascribed to the properties of the glandular epithelium the vascular system and the blood it carries to the epithelium and the manner in which these interact

The most common theory offered to explain gas secretion was first proposed by HALDANE (1922) It postulates in its most complete version (KUHN and KUHN 1961) that some acid substance is released into the blood as it passes through the swimbladder This acid causes an increased O tension in the blood (Bohr Root effect) and an increased tension of gases in general due to the salting out effect of the acid A gas tension gradient is hereby induced between efferent and afferent blood in the vascular counter current bundle the *rete* of the gas gland In this *rete* the primary gradient is then multiplied to considerable pressures allowing gas to enter the bladder by diffusion

It is clear that a knowledge of the properties of fish blood is important for our understanding of both reabsorption and secretion of gases It seemed appropriate therefore to begin this study of the mechanisms of gas exchange in the swimbladder of the eel with an analytical investigation of some pertinent factors which influence the content of atmospheric gases in the eel blood

When the blood of most fishes is acidified by CO_2 or lactic acid it exhibits not only a Bohr effect *i e* a lowering of the O_2 affinity with full oxygenation still obtainable at high enough pO but it apparently also suffers a loss of O_2 capacity (KROGH and LEITCH 1919 ROOT 1931 POWERS 1932 SCHOLANDER and VAN DAM 1954) This property of fish blood is called the Root effect SCHOLANDER and VAN DAM (1954) found a pronounced Bohr effect in the blood of several species of deep sea fishes where the hemoglobin at low pH attained full saturation only at pO_2 between 20 and 130 atm In other fishes pO_2 up to 140 atm did not increase the hemoglobin bound O_2 above that recorded at 20 atm indicating strongly that the blood suffers a permanently reduced O_2 capacity (Root-effect) These experiments also make it clear that one cannot decide on the presence of a Root effect based on low pressure observations alone

VAN SLYKE, DILLON and MARGARIA (1934) found no effect of pH variations upon the solubility of N in ox blood They showed that N_2 dissolves according to Henry s law The same has been shown to be true for N_2 in the blood from two species of physostome fishes (*Coregonus lavaretus* and *Salmo alpinus*) (SUNDNES ENNS and SCHOLANDER 1958)

The content of CO_2 in blood is closely related to the buffering capacity In most cases fish blood has a lower CO binding power and a lower buffering capacity than has mammalian blood (ROOT 1931 REDFIELD 1933 [illegible] and BLACK 1941)

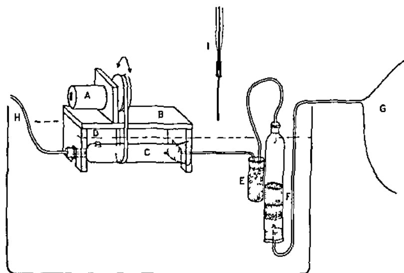


Fig 1 Blood gas equilibrator A — Window cleaner motor B — rack C — tonometer D — rubber stopper for blood samples E — drop catcher F — humidifier G — gas filled rubber bladder I — blood pipette with modified hypodermic needle and H — gas exit The water level is indicated by the broken line The chamber is kept in position by the rubber band, which also acts as a driving belt During sampling the chamber is pushed down and out of the rack so that the rubber stopper is just above the water surface

Although previous investigations (KAWAMOTO 1929 I and II RIGGS 1951) strongly indicate that eel blood is generally similar to blood from other fishes a detailed knowledge of some of its reactions was necessary The main object of this investigation was to establish the effect of varying pH and temperature upon the relation between the content and the tension of the atmospheric gases in eel blood This enabled estimation of the gas tension from measurements of gas content pH and temperature in later *in vivo* experiments where direct tension measurements were difficult In addition the buffer capacity of eel blood at different CO_2 tensions has been investigated

Material

These investigations were performed mainly on blood from the eel *Anguilla vulgaris* but also to a small extent on blood from codfish *Gadus callarias* rainbow trout *Salmo irrideus* and man

Female eels were bought at the fish market They had been captured in salt or brackish water At the laboratory they were kept in running fresh water The temperature of the water varied from 5 to 15 C depending on the time of the year Although the eels were not fed they kept in good condition for several months Codfish captured in the Oslofjord were kept in aerated seawater at 12 C Rainbow trout were bought from a trout breeder and kept in running tap water at 10 C Cod and trout were kept for only a few days in the tanks Human blood was supplied by the author

The fishes were bled by heart puncture The blood was drawn into a heparinized syringe and strained through a double layer of gauze before use Blood was always used the same day it was drawn The equilibration gases were mixed from out door air purified O_2 N_2 and Ar and technical CO_2

Methods

An average eel gives 2—4 ml of blood As this sample should provide adequate amounts for several different analyses it was necessary to develope techniques for equilibration of small volumes of blood and to apply micro methods

1 *Blood gas equilibration* A tonometer was constructed for this part of the experiments This instrument is shown schematically in Fig 1 It consists of a horizontal tube which is rotated approximately 250 back and forth around its long axis while flushed with gas from a reservoir The gas is bubbled through a solution of isotonic NaCl before it enters the equilibration chamber Atmospheric pressure prevails inside the chamber during equilibration Except for the gas source and the gas outlet the entire instrument is immersed in a waterbath thermoregulated to within 1 C With this technique as little as 0 5 ml blood can be equilibrated for 12 hours without any measureable change in blood volume or any signs of hemolysis

Blood samples were sucked up into the appropriate pipettes through the hole at *D* while the gas outlet was temporarily clamped During this sampling the tonometer was lifted out of its holder *B* so that the stopper in *D* was above water level. Gas samples were taken at *H*

2 *Blood titration* A 2 5 ml blood sample was poured into a 5 ml vial with a plastic cover A pH electrode and a glass tube leading gas from a reservoir entered the vial through two holes in the cover The glass tube was drawn out to a fine tip which sent a stream of small bubbles through the blood The gas escaped through a third hole in the cover The titration was done by measuring the change in pH caused by stepwise additions of 0 17 M lactic acid or HCl Usually 5 or 10 min were necessary for the pH to stabilize after addition of acid In some experiments a drop of octyl alcohol was added to prevent foaming This procedure allowed estimation of the buffer capacity of blood which was continuously equilibrated with gas of a known pCO_2

3 *Analytical Methods* The content of O_2 in 40 μl blood was estimated by the method of ROUGHTON and SCHOLANDER (1943) with the modifications prescribed for fish blood by SCHOLANDER and VAN DAM (1956) The further modifications described by SUNDNES ENNS and SCHOLANDER (1958) for blood from some salmoid fishes turned out to give unsatisfactory results on eel blood Duplicate analyses on eel blood agreed within 0 4 vol %

The content of CO_2 in about 13 μl blood was estimated by the method of SCHOLANDER and ROUGHTON (1943) Duplicate analyses on eel blood agreed within 0 7 vol %

The content of N_2 and Ar in 120 μl blood was estimated by the method of EDWARDS SCHOLANDER and ROUGHTON (1943) with one modification The acid solution of the O_2 method for fish blood was used instead of that recommended This proved necessary in order to break the blood into flocculi Duplicate analyses on eel blood agreed within 0 05 vol %

Gas samples were analysed with the 0 5 ml apparatus (SCHOLANDER 1947) Duplicate analyses agreed within 0 03 vol

The pH of 25 μl blood was measured with a Methrom pH meter equipped with a capillary micro blood electrode The meter was checked prior to each measurement with a buffered solution of pH 7 12 The pH of duplicate blood samples agreed within 0 02 units All determinations were done at room temperature (19 to 22 C) For these

measurements blood was transferred from the pipette to the pH electrode in th following way A drop of blood was deposited on a clean and hydrophobic porcelain dish The tip of the electrode was put into the center of the drop and the blood was sucked into it The blood which came in contact with the electrode was thereby obtained from the center of the drop which did not suffer any significant loss of CO_2 during the very short time at most 15 sec during which the drop was in contact with air The dependability of this procedure was established by comparing the pH of samples from th same blood transferred anaerobically with samples transferred as described These values never deviated more than 0 03 units from each other

The content of lactate was estimated by a micro diffusion method (CONWAY 196?) modified by SCHOLANDER and BRADSTREET (1962) to allow analysis on blood samples as small as 5 mg Duplicate analyses on the standard agreed within 4 mg/100 ml and on blood samples within 6 mg/100 ml Analysis on known dilutions of the standard revealed a direct proportionality between lactate content and optical density

Hematocrit was determined either in a conventional hematocrit tube or simply in the open blood pipette centrifuged with its upper end in a drop of mercury The values were read after 20 min centrifugation at 850 g Duplicate determinations agreed within 1 5 %

Experimental Procedure

Blood gas equilibration The content of O_2 at different pO_2 and pH levels was studied as follows Blood to which varying amounts of 0 17 M lactic acid had been added was equilibrated with a mixture of O_2 and N_2 After 30 min equilibrium was established and a gas sample was analysed for O_2 and inert gases Blood samples were analysed for O_2 after 40 and 50 min Additional samples were taken for determination of pH and hematocrit This procedure was sub equently repeated without further additions of acid with 4 to 6 different mixtures of N_2 and O_2 Experiments of this kind covering a pH range from about 8 6 to 6 6 were performed at 8 14 and 19 C.

The experiments on the content of CO in eel blood were arranged very similarly to which varied amounts of 0 17 M lactic acid had been added was tonometered ith a mixture of O_2 and CO_2 Gas and blood samples were analyzed after at least 30 min of equilibration Determinations of pH and lactic acid content (measured as lactate) were performed on parallel samples The procedure was repeated on 4 to 6 different gas mixtures but without further additions of lactic acid Experiments were performed at 6 5 and 19 5 C

Two procedures were applied to study the solubility of N_2 and Ar 1) Blood was equilibrated with N_2 (or Ar) either pure or admixed with CO_2 and/or O_2 In this way both pH and pN_2 (or pAr) was changed from experiment to experiment 2) Blood was equilibrated with N_2 (or Ar) either pure or admixed with O_2 The pH was changed by adding 0 17 M lactic acid or 0 17 M NaOH to the blood Such experiments with N_2 were performed on eel blood at 6 5 and 19 5 C on eel plasma at 6 5 C and on blood from cod trout and man at 6 5 C Ar solubility was investigated only on eel blood and at 6 5 C

Blood titration A 5 ml blood sample was divided in two halves One was temporarily stored in the refrigerator The other was introduced into the titration chamber and equilibrated with CO_2-free air After one hour titration was started The other sample was equilibrated with air containing 3 % CO_2 and 18 % O_2 and then titrated In other experiments the first sample was equilibrated with CO_2-containing air and the second with CO_2 free air The same procedure was used on blood which had received a drop of octyl alcohol Both types of blood were titrated with both lactic acid and *HCl* Such series of titrations were performed on eel blood and on human blood at 20 C

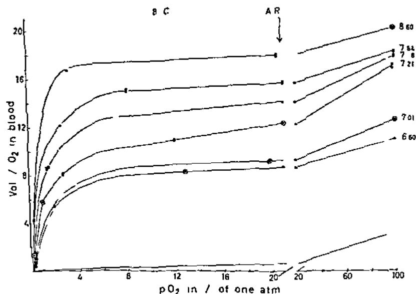


Fig 2

Fig 2 3 and 4 show the total O content (hematocrit 40 /) vs pO at various pH levels of whole blood at 8 14 and 19 C The pH of the blood equilibrated with a r is given by the numbers to the right of each curve The bottom curve in fig 2 and 3 gives the measured O_2 content of the plasma

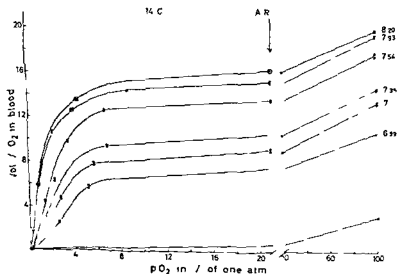


Fig 3

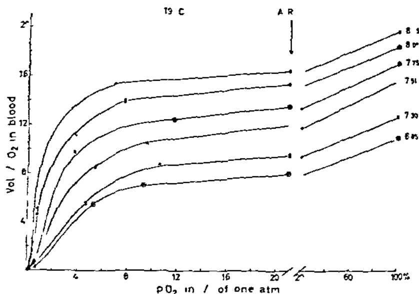


Fig 4

Results

The content of O_2 Under identical conditions of pO pH and temperature the O_2-content of the blood varied according to the hematocrit which was between 30 and 50 % This indicates that the hematocrit is proportional to the Hb-content of the blood To standardize the results all of the values for O_2 content have therefore been adjusted to hematocrit 40 % This was done by subtracting the physically dissolved O_2 from the measured O_2-content and regarding the remaining O_2 as proportional to the measured hematocrit The dissolved O_2 was then added to the amount of O_2 which was calculated to be chemically bound to blood with hematocrit 40 % The data represents therefore total O -content which would have been found had the hematocrit been 40 %

In Fig 2 3 and 4 the O_2-content of blood at 8 14 and 19 C is plotted against the O_2 pressure The latter is given in % of 1 atm The atmospheric pressure during these experiments varied between 740 and 770 mm Hg and the yearly variation in Oslo is usually within 730 and 775 mm Hg The small errors introduced in this way are insignificant in the present context

The curves in Fig 2 to 4 are sigmoid as can be best seen in those taken at low pH values The pO_2 which corresponds to the point on the curve where it attains the slope for dissolved O_2 is interpreted as the saturation point of the hemoglobin at that pH

The effect of temperature upon the O_2-capacity is dependent both on the pO_2 and pH The lower the temperature the lower is the pO_2 at which the

6 5 C

Total CO_2 in blood vol /

pCO_2 in / of one atm

Fig 5

Fig 5 and 6 Total CO_2-content vs pCO_2 at various pH levels of whole eel blood at 6 5 and 19 5 C Broken curves are iso-pH curves with pH of each curve on top Numbers of the right of each curve give the lactic acid content in mg %

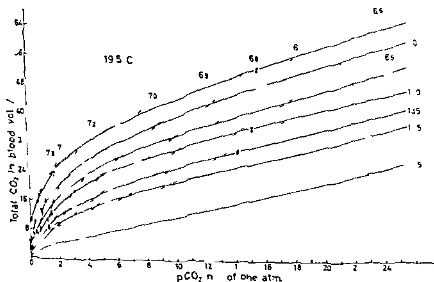


Fig 6

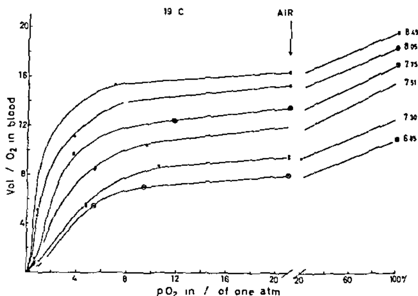


Fig 4

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The content of O_2 Under identical conditions of pO_2 pH and temperature the O content of the blood varied according to the hematocrit which was between 30 and 50 % This indicates that the hematocrit is proportional to the Hb content of the blood To standardize the results all of the values for O_2 content have therefore been adjusted to hematocrit 40 % This was done by subtracting the physically dissolved O_2 from the measured O_2 content and regarding the remaining O_2 as proportional to the measured hematocrit The dissolved O_2 was then added to the amount of O_2 which was calculated to be chemically bound to blood with hematocrit 40 % The data represents therefore total O_2 content which would have been found had the hematocrit been 40 %

In Fig 2 3 and 4 the O_2 content of blood at 8, 14 and 19 C is plotted against the O_2 pressure The latter is given in % of 1 atm The atmospheric pressure during these experiments varied between 740 and 770 mm Hg and the yearly variation in Oslo is usually within 730 and 775 mm Hg The small errors introduced in this way are insignificant in the present context

The curves in Fig 2 to 4 are sigmoid as can be best seen in those taken at low pH values The pO_2 which corresponds to the point on the curve where it attains the slope for dissolved O_2 is interpreted as the saturation point of the hemoglobin at that pH

The effect of temperature upon the O_2 capacity is dependent both on the pO_2 and pH The lower the temperature the lower is the pO_2 at which the

Table I Solubility of N_2 in blood from eel cod trout and man at 6.5 C the solubility of N_2 in eel blood at 20 C and the solubility of Ar in eel blood at 6.5 C

Species	No of animals	Temp C	pH range	Vol % N_2 at 1 atm N_2 average (spread)	Vol % Ar at 1 atm Ar average (spread)	No of analyses
Eel	1	20	6.60– 8.50	1.49 (1.46–1.55)	–	10
Eel	8 } same	6.5	≈7.60– 8.50	1.81 (1.71–1.85)	–	19
Eel	8 } blood	6.5	6.20–≈7.60	1.65 (1.57–1.73)	–	19
Eel	2 } same	6.5	≈7.90– 8.50	–	3.93 (3.89–4.01)	8
Eel	2 } blood	6.5	6.50–≈7.90	–	3.72 (3.70–3.73)	6
Cod	2	6.5	6.40– 8.50	1.66 (1.62–1.68)	–	10
Trout	2	6.5	6.40– 8.50	1.74 (1.64–1.75)	–	12
Man	1	6.5	6.40– 8.60	2.04 (2.00–2.11)	–	10

pH around 8.5 when equilibrated with pure O_2. Under the same conditions blood from eels more recently caught in salt water showed a lower pH down to 7.5 in freshly caught individuals. This difference in pH was not due to a different lactic acid content. The lactic acid values given in Fig 5 and 6 apply only to "fresh water eels". These curves can be used therefore to obtain a reasonable accurate estimate of any two of the four mentioned parameters when the other two are known. For the blood from "salt water eels" the curves can be used to estimate any one of the parameters pCO_2, CO_2-content and pH when the two others are known.

The curves in Fig 5 and 6 also give information about the buffer capacity of the blood. This aspect will be discussed in connection with the titration experiments.

The solubility of N_2 and Ar. When the pH of eel blood at 6.5 C was lowered stepwise by addition of lactic acid the N_2 solubility was constant down to a pH of about 7.5 to 7.7. At this point a sudden fall in N_2 solubility was observed. If pH was again raised by NaOH the N_2 solubility also increased. The same was observed when the pH was varied by adding CO_2 to the equilibrium gas. The pH value at which N_2 solubility was observed to suddenly decrease varied from specimen to specimen but was always between 7.5 and 7.9. Typical curves are shown in Fig 7. This effect of pH was found in the blood of 6 eels while in blood from 2 others it was so small that it could possibly have been due to measuring errors. The same effect of pH was also found when the solubility of Ar in eel blood was investigated (Fig 7).

Exactly the same procedure was applied to study the effect of pH upon the solubility of N_2 in plasma of eel blood, blood from cod, trout and man at 6.5 C and on eel blood at 20 C. In none of these cases was the solubility of N_2 found to be measurably influenced by the pH of the blood. The results of these tests are compiled in Table I.

milieu as an eel changes environment during its life span The eel is born in the Sargasso sea migrates to fresh water lakes and streams wherefrom it again undertakes its much studied but poorly understood migration back to the spawning grounds The results of the present study throw little light on the properties of eel blood in its natural environment They have, however proved valuable in further inquiries into the mechanism of gas reabsorption and gas secretion in the swimbladder under laboratory conditions

During this investigation I have benefited by professor R FÄNGE s thorough familiarity with the physiology of the swimbladder I am indebted to professor P F SCHOLANDER for his criticism of the manuscript I also wish to acknowledge the expert craftmanship of mr O IVERSEN at our laboratory workshop and valuable suggestions from my brother H STEEN I wish to acknowledge financial support from A/S Norsk Varekrigsforsikrings Fond

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From the Institute of Zoophysiology, University of Oslo Blindern Norway

The Physiology of the Swimbladder in the Eel Anguilla vulgaris

II The reabsorption of gases

By

Johan B Steen

Received 27 October 1962

Abstract

Steen J B *The physiology of the swimbladder in the eel Anguilla vulgaris* II The reabsorption of gases Acta physiol scand 1963 *58* 138—149 — Gas reabsorption from the reabsorbent swimbladder has been investigated with the micro-blood analyzer and gas analyzer of Scholander and coworkers It was shown that O_2 moves both from blood to gas and *vice versa* The rate of O_2 reabsorption depends upon blood flow and the O_2 gradient between gas and blood All the observations support the view that O_2 is reabsorbed by diffusion Blood flow is much increased by anoxia The reabsorbed O_2 is hardly of any respiratory use during O_2 shortage of the water but may be important when the eel is in the air The increased O_2 reabsorption during O_2 shortage may also guide the eel to a more O_2 rich environment Fishes have less hydrostatic problems the deeper they live The reabsorbent bladder of the eel seems to have about the same diffusion characteristics as has the human lung the functional superiority of the latter beeing due primarily to its favourable surface to volume ratio

Fishes obtain neutral buoyancy with the surrounding water by means of the gasfilled swimbladder (Borelli 1679) Moreau (1876) showed that the bladder volume is regulated by its content of gas while the pressure inside it is equal to the hydrostatic pressure of the environment He thus established that a fish maintains neutral buoyancy by an interplay between secretion and reabsorption of gases

Many fishes have a particular area of the swimbladder specialized for gas reabsorption This area is supplied by arterial blood (WOODLAND 1911, VON LEDEBUR 1937 FANGE 1953, DENTON 1961 DORN 1961) where the tension of the atmospheric gases is approximately equal to their partial pressure in air at the surface Since the total pressure of gases in the bladder is above one atm, there will always be a tendency for gas to diffuse into the blood In accordance with this VON LEDEBUR (1929) demonstrated that the reabsorbent bladder of *Serranus cabrilla* became completely emptied within a week after it had been separated from the secreting bladder by a ligature MOREAU (1876) suggested that gas reabsorption occurs by diffusion of gases into the blood, and this view has not since been questioned

The swimbladder gas from fishes living in well aerated surface water usually has a composition similar to that of air although O_2 pressures down to 0 01 atm have been recorded SUNDNES (1959) *e g* overinflated the bladder of a codfish with a gas containing 74 % O_2 and 23 % N_2 Four days later the fish showed normal buoyancy and a swimbladder gas consisting of 96 % N_2 and 4 % O_2 MEESTERS and NAGEL (1935) and ROSTORFER (1942) found that CO_2 was most easily reabsorbed whereas O and in particular N_2 were reabsorbed more slowly FANGE (1953) partially anaesthetized *Ctenolabrus rupestris* by replacing some of the gas in the swimbladder with CO_2 while the fish was stimulated to reabsorb

In the eel gas reabsorption occurs in a special bladder which is separated from the secretory bladder by a tube The aim of this investigation was to describe the factors which influence the rate of O_2 reabsorption in the eel bladder This includes measurements of O_2 reabsorption and O_2 tension in arterial and venous blood of the bladder under selected conditions

The results make possible a rough comparison between gas exchange as it occurs in the reabsorbent bladder and in the vertebrate lung The two organs which both serve a rapid gas exchange between blood and gas also have structural similarities which make this comparison valid DORN (1961) has given a detailed picture of the fine structure of the swimbladder in the eel The epithelium lining the reabsorbent bladder is flat and the tissue as a whole has a striking resemblance to alveolar capillary walls in lungs (DE GROOT 1961)

Material

The investigation was done on female specimens of the eel *Anguilla vulgaris* kept in running tap water which had a temperature from 5 to 17 C depending in the time of year

The eel is a physostome with the swimbladder connected to the foregut by an open muscular tube the pneumatic duct This duct does not enable the eel to fill the bladder by swallowing air When the pressure inside the bladder exceeds the atmospheric pressure however gas may escape through this passage

Fig 1 shows a drawing of the swimbladder The pneumatic duct (or reabsorbent bladder) has a muscular sphincter at its junction with the gut. From this point it extends

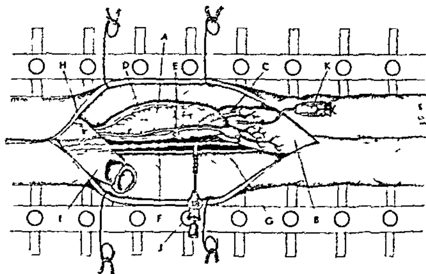


Fig 1 The swimbladder of the eel in its experimental position Natural size A = reabsorbent bladder (pneumatic duct) B = secretory bladder C = rete mirabile D = pneumatic duct vein, E = swimbladder artery F = swimbladder vein G = dorsal vein H = sphincter closing the opening to the gut I = gut, J = cannula in dorsal artery K = cannula into reabsorbent bladder The eel is immobilized in the eel holder

posteriorly between the gut and the kidneys It is very vascular and constitutes the only area of the swimbladder specialized to reabsorb gases The duct connects to the secretory bladder about midway along its length via a narrow tube which lies between the two counter current capillary bundles called *retia mirabilia* This makes a very convenient place for a ligature to separate the two bladders Both bladders receive blood from a common artery but the venous return is through separate veins MOTT (1950) has shown that blood can be shunted separately through either bladder

Methods

Analytical methods

Most of the analytical methods employed are described in more detail by STEEN (1963a) Gas composition was analysed using the 0 5 ml analyzer (SCHOLANDER 1947) or when only small samples were available with the water analyzer (SCHOLANDER *et al* 1955) The former gives the O_2 and CO_2 with an accuracy within $\pm$ 0.0[illegible] the latter within $\pm$ 0.2 %

Forty μl samples of blood were analysed for O_2 with the ROUGHTON and SCHOLANDER (1943) syringe technique modified for fish blood after SCHOLANDER and VAN DAM (1956) Repeated analyses agreed within 0 4 vol %

The acidity of 20 μl blood samples was measured within 0 02 units with a Metrohm pH meter equipped with a special micro blood-electrode

Hematocrit values were determined either with a clinical hematocrit tube or simply in the blood pipette centrifuged with its upper end in a drop of mercury

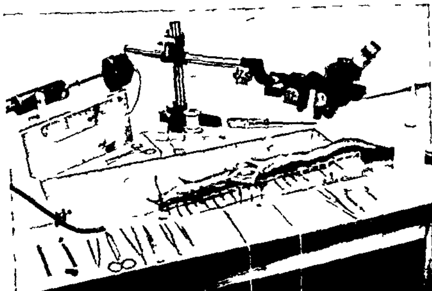


Fig 2 The experimental arrangement with eelholder water supply binocular microscope and sample storer

Experimental

An *eel holder* held the fish belly up between two rows of pointed steel bolts Tapwater was supplied through a glass tube tied into the mouth From the gills water flowed down into a bucket where it was aerated and pumped back to the eel. Fig 2 illustrates some essential features of the experimental arrangement

Isolation of reabsorbent bladder A hypodermic needle was inserted into the reabsorbent bladder to establish access to its contents The head of the needle was closed with a rubber stopper and the tip was pushed into a tight fitting plastic tube slightly heated at the tip to make a collar This cannula was introduced through a hole in the secretory bladder pushed into the other bladder and then fastened with a ligature around the duct connecting the two bladders (Fig 1) The collar prevented the cannula from slipping out and secured a tight connection The contents of the bladder were anaerobically reached by pushing a hypodermic needle fitted to a syringe through the rubber stopper in the needle head

The *rate of oxygen uptake* from the gas in the isolated reabsorbent bladder was quantitatively determined in the following way A gas sample was introduced into the bladder from a glass tuberculine syringe fitted with a modified hypodermic needle The head of this needle was cut in the lathe until it could be fitted to the syringe by a short length of plastic tubing The gas was pushed back and forth a few times and a 0 05 ml sample anaerobically transferred to the analyzer 10 or 15 minutes later the gas was withdrawn into an identical syringe the dead space of which was filled with a saturated solution of citric acid The volume of the sample was measured with a liquid seal in the tip of the syringe so that atmospheric pressure prevailed inside the syringe Repeated withdrawings of the same sample agreed to within 0 0? ml A sample of the gas was analysed These measurements enabled calculation of the O_2 uptake within 0 03 ml

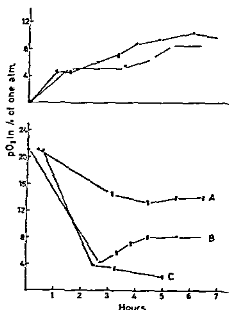


Fig 3 Variation in the composition of the gas in the reabsorbent bladder In some experiments the bladder was initially filled with pure N_2 (upper curves) and the eel had ample water supply In others the bladder was filled with air (lower curves) Curve A represents the results from an experiment where the eel had ample water supply in curve B it had a somewhat restricted water supply and in curve C it received almost no water

Blood samples were taken by puncturing the appropriate vessel with a modified no 20 hypodermic needle attached to a calibrated blood pipette as described above Prior to use a drop of heparine was sucked through the pipette and dried by suction During sampling the blood filled the pipette by its own pressure

Very often several blood samples were taken consecutively and a sample-storer was constructed to minimize changes while they waited to be analyzed or treated further This sample-storer is shown in Fig 2 It rotates 180 back and forth to prevent sedimentation and is cooled by tap water to avoid supersaturation of the blood

Results

Composition of the gas in the reabsorbent bladder

The swimbladder gas of several eels tested showed large variation The O_2-content varied from 2 to 70 % the CO_2 from 1 to 7 % and the N_2 from 90 to 30 % of one atm

In the first series of experiments the reabsorbent bladders of 11 eels were cannulated and separated from the secreting bladder The reabsorbent bladder of each was then filled with N_2 or air and the fish sutched together and returned to the aquaria At varying periods of time eels were quickly anaesthetized and opened The reabsorbent bladder was always almost entirely empty the residual gas having a composition from 1 to 4 % O_2 1—2 % CO_2 and the rest N_2.

In another series of experiments eels were operated as above but kept on the table while they received water at varying rates The isolated reabsorbent bladder was filled with 1 ml of either N_2 or air and the variation in composition followed by periodic analysis of small gas samples

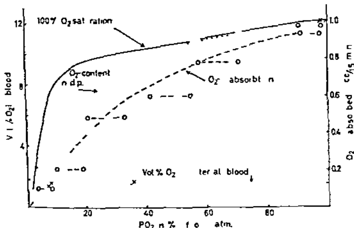


Fig 4 compares the rate of O reabsorption to the O content of the blood leaving the bladder The degree of O saturation of blood circulating the bladder is illustrated by comparison of the latter curve to the O_2 saturation curve

Fig 3 gives some typical curves It is evident that the final gas composition is independent of the initial composition but dependent upon the water supply In eels with ample water supply the O_2 content stabilized at about 0 12 to 0 14 atm in 5 hrs This indicates that the O_2 tension of the arterial blood was about 0 13 atm When the bladder initially contained N_2 the O_2 gradient from blood to bladder was therefore about 0 13 atm — when it was filled with air it was about 0 08 atm in the opposite direction The fact that the initial 1 ml gas sample attained the same composition during the same time interval in dicates that the rate of O_2 movement is within the same order of magnitude in both directions

The rate of O_2-reabsorption

The rate of O_2 reabsorption was shown to be influenced by 1) the O_2 gradient between blood and gas 2) the O_2-capacity of the blood supplying the bladder and 3) the blood flow through the bladder

The effect of varying O_2 gradients upon the rate of O_2 reabsorption was studied in the following way The water supply to an eel was reduced so that the arterial blood attained less than 20 % saturation The isolated pneumatic duct was repeatedly flushed with an O_2—N_2 mixture of known composition 1 ml of which was left in the bladder 1 or 2 min later blood samples were taken first from the pneumatic duct vein and then from the bladder artery Finally a blood sample was taken from the dorsal artery The samples were stored for less than 15 min before they were analyzed for O_2 Hematocrit was determined on arterial blood In parallel experiments the rate of O_2 reabsorption from gas mixtures of varying O -content was determined

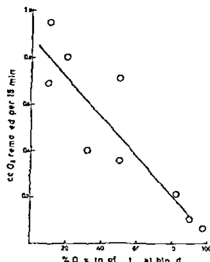


Fig 5 This diagram shows the rate of O_2 reabsorption versus the O_2 saturation of the blood entering the bladder

In Fig 4 the results are plotted and compared with the O_2 equilibrium curve at the prevalent pH 7 2 (This curve was obtained with methods described in STEEN 1963 a) *One observes that 100 % saturation of the* blood occurs only when the pO_2 in the bladder is above 0 5 atm At the pO_2 of air for example the degree of oxygenation is slightly below 70 % The rate of O_2 reabsorption at varying pO_2 in the gas follows approximately the amount of O_2 combined with blood indicating that the blood flow is rather constant

The effects of O_2-capacity and blood flow were studied in the following way An eel lying in the holder was equipped with the usual cannula in the re absorbent bladder The O_2 pressure in the blood was changed either by varying the water flow or by bubbling more or less pure N_2 through the water 75 min after regulation of the water supply and/or N_2 bubbling 1 ml pure O_2 was introduced into the pneumatic duct and a measurement made of the amount of O_2 taken up during 15 min The bladder was again filled with pure O_2 10 min later After 2 min blood samples were taken in the following sequence from the heart from the vein leaving the bladder from the dorsal vein and from the dorsal artery Not more than 10 min elapsed from starting the first sample to finishing the last one These samples measuring about 60 μl each were analyzed for O_2 and pH An additional sample was taken from the dorsal artery for hematocrit

The results of these experiments are presented in Fig 5 and in Table I In Fig 5 the rate of O_2-reabsorption is plotted against percent O_2-saturation of arterial blood 100 % saturation represents the O_2-content of blood with the pH of the arterial blood and saturated with air These values are taken from an earlier work (STEEN 1963a) The O_2-reabsorption under these conditions

Table I shows the effect of anoxia upon 1) the O_2 carrying capacity of blood entering the bladder and 2) the proportion of total blood flow which circulates the bladder. It also shows the degree of equilibration between O_2 in the bladder and in the blood

Blood entering the pneumatic duct				Blood leaving the pneumatic duct		% of blood in heart from pneumatic duct
pH	O_2-cont Vol	% satn	O_2-cap Vol %	O_2-cont. Vol	% satn	
7.24	1 3	10	11.2	12 5	100	18
7 13	2 0	20	8 5	10 5	95	28
7 75	4 0	30	12 5	16 5	100	25
7 76	6 [illegible]	50	9 3	15 5	90	15
7 77	7 5	50	9 0	16 5	100	15
7 56	10 0	80	6 0	16 0	100	6
7 87	13 5	100	3 5	17 0	100	10
7 50	12 0	100	3 0	15 0	90	12

[1] 100 is O_2 content at 0 2 atm O_2
100 is O_2 content at 1 0 atm O_2

of pure O_2 in the bladder increases by 10 fold as the degree of blood saturation falls from 100 % to 10 %

The absolute blood flow through the pneumatic duct obtained by dividing the O_2 reabsorbed with the A—V O_2 difference varied considerably due to varying size of the swimbladders. The relative blood flow (*i e* the proportion of the total flow going through the reabsorbent bladder) could also be calculated since the O_2 content measured in the mixed heart blood is the sum of the O_2 in venous blood and that from the reabsorbent bladder both of which were measured. Table I shows that the relative blood flow through the pneumatic duct increases with increasing deoxygenation of the blood. These values suffer a rather large uncertainty both due to measuring error and to the fact that the proportion of the two venous returns to the heart may vary so that the rather short sampling time may not be representative

The table shows further that when the bladder contains pure O_2 almost deoxygenated arterial blood can carry 3—4 times more O_2 than can blood which is fully saturated at 0 2 atm O_2. The O_2 content of the venous blood leaving the bladder corresponds very closely to the saturation values at 1 atm O_2 presented earlier (STEEN 1963a)

When the reabsorbent bladder was inspected through a binocular microscope it was observed that as anoxia developed blood started to circulate in an increasing number of capillaries. The blood pressure in the bladder vein judged by the time needed for the blood to fill the sampling pipette was clearly lower during anoxia than under normal conditions. Although these

observations are only qualitative, they indicate that the increased flow through the reabsorbent bladder is accompanied by general vasodilatation. This means that the exchange area between blood and gas increases.

Discussion

The results are all in agreement with the view that O_2 is reabsorbed from the bladder solely by diffusion, although conclusive evidence for this is not presented. The observation that O_2 can move from bladder to blood and *vice versa* at rates which are not radically different and that the O_2 tension in the venous bladder blood never exceeded the pO_2 in the bladder in cases when the pO_2 was higher than the arterial O_2 tension is strong evidence in favour of this view. It is further corroborated by the fact that the rate of O_2 reabsorption can be accounted for by the gradient in O_2 tension between bladder and blood together with the O_2 capacity of the arterial blood and the blood flow.

It is very likely therefore that the mechanism of O_2 exchange is the same in this bladder as it is in the vertebrate lung. GOODRICH (1930) suggests that these two organs may indeed be homologues although the evidence is ambiguous. We may therefore with some justification consider the reabsorbent bladder as a primitive edition of the vertebrate lung.

The diffusion properties of the human lung and the reabsorbent eel bladder may be compared on the basis on their diffusing capacities (D_0). In respiratory physiology this term is defined as the volume of O_2 which diffuses from the alveolar gas to blood per min and mm Hg pressure gradient under conditions where the blood leaving the lungs is not saturated at the pO_2 of the gas (FORSTER 1957). The D_0 for the human lung varies for several reasons but 50 ml/min mm Hg seems to be a reasonable average value. An average value for the diffusion area of the lung is 50 m². The D_0 per cm² becomes therefore $50/50 \times 10^4 = 1 \times 10^{-4}$ ml/min mm Hg cm². We can obtain an approximate value for the D_0 of the reabsorbent bladder from the data in Fig. 4. From a gas containing 35 % O_2, 0.5 ml O_2 was reabsorbed during 15 min, and the final gas contained 20 % O_2. The average pO_2 of the gas is therefore 0.275 atm. The blood leaving the bladder contained 8 vol% O_2, corresponding to an O_2 tension of 0.08 atm. The average gradient between blood and gas is therefore $0.275 - 0.045 = 0.230$ atm (the blood enters the bladder with an O_2 tension of 0.01 atm) or 175 mm Hg. The D_0 becomes $\frac{0.5}{15 \times 175} \approx 2 \times 10^{-4}$. The area of this bladder is about 6 cm² which makes the D_0 per cm² 0.3×10^{-4}, i.e. about one third of the value for the human lung. The diffusion distance between gas and blood in the lung is commonly set to 1.5 μ. The thickness of the comparable path in the eel *reabsorbent bladder can be measured* in electronmicrographs of DORN (1961) to be from 4 to 5 μ. The diffusion path is

therefore some 3 times longer in the pneumatic duct than in the human lung The difference in D_0 per cm^2 seems therefore to be due to the difference in length of diffusion path

Although there are large uncertainties in these values they indicate that the permeability to O_2 for the two tissues are approximately the same and also that the vascularization per unit area is about equal The main difference between the two organs seems therefore to be the architecture of the lungs which involves a large diffusion area compared to the total volume

In many fishes especially swamp dwelling species the swimbladder functions as a lung which is periodically ventilated at the surface (HARDEN JONES and MARSHALL 1953) Most fishes, however do not or cannot ventilate their bladders In these cases the O_2 in the bladder may serve only as an O_2 store Experiments have shown that the bladder O_2 in fishes living at low pressures will last for no more than 5—15 min (HARDEN JONES and MARSHALL 1953) In deep sea fishes however both the proportion of O_2 and the density of the gas is greater It is possible therefore that O_2 stored in the bladder may be of significance in these fishes although accumulation of metabolic CO_2 in the blood may become prohibitively high

A necessary prerequisite for this utilization however is that the O can be circulated to the tissues Most teleosts are euphysoclists and the vein from the resorbing area carries blood directly to the heart (FANGE 1953 MARSHALL 1960) Here the blood is mixed with venous blood and circulated to the gills These are very efficient equilibrators and the blood will leave them with the pO_2 of the water During mild O_2 shortage the O_2 which is reabsorbed in the bladder will partially saturate the blood before this enters the gills During more pronounced O_2 shortage — when the bladder O_2 would be most useful — the pO_2 in the water may be lower than the pO_2 in the blood and the fish will loose O_2 to the water The fish could possibly prevent this loss by restricting the circulation of water in the gill cavity and/or by decreasing the exchange efficiency of the gills It has frequently been observed however that fishes respond to O_2 shortage by hyperventilation (KROGH 1940) Exchange efficiency could be reduced by vasoconstriction of the capillaries of the gills together with arterial venous shunts There are no anatomical evidence of such shunts and the vasoregulation of the gill capillaries in response to O shortage has not been investigated

It would appear therefore that the bladder O_2 of euphysoclists (and other fishes with a similar vascular anatomy) cannot reach the other tissues hence it has no respiratory significance The observed increased O_2 reabsorption during anoxia may on the other hand have some ecological significance

HOGLUND (1961) showed that some fishes seek out the most O_2 rich water of an environment of graded O concentration simply by increased random swimming activity in the O_2 poor areas In the eel the O_2 poor environment also induces increased O_2 reabsorption in the swimbladder These two reac

tions will if the eel steadily seeks a depth where it is neutrally buoyed, lead the fish closer to the surface where the O_2 content is always high

Compared to other fishes the eel can stay on land for a long time They live for at least 24 hrs on the moist cement floor of the aquaria room in our laboratory Under such conditions one can observe that the gill cavity is kept blown up but closed Only rarely does the eel renew the air in the gill cavity Under these conditions therefore the eel can utilize the O_2 which is reabsorbed from the bladder in respiration It is also probable that the exchange efficiency of the gills are reduced when they are surrounded by air and not water since the lamellae of the gills will stick together

When a fish swims to greater depths gas secretion is desirable to save the work needed to avoid sinking but lack of secretion does not have fatal results When swimming upwards however, the swimbladder will expand, which may be fatal as commonly observed Different species are certainly able to tolerate different degrees of bladder expansion But since the volume will increase as the ratio of displacement — in terms of pressure — over the initial pressure, fishes will have more vertical freedom the deeper they live

It seems reasonable to assume that fishes regulate gas reabsorption mainly according to hydrostatic necessity while the reaction to anoxia is more secondary Reabsorption for volume regulation probably usually occurs in well aerated water hence the blood reaching the reabsorbent bladder has a pO_2 close to 0 2 atm As a consequence O_2 reabsorption under these conditions is not dependent on the capacity of hemoglobin to carry O_2 unless its O_2 dissociation curve is sigmoid or otherwise unnormal This means that all the gases of air are reabsorbed according to their pressure gradient and their solubility in blood and in the tissue separating blood from gas

[1] I am thankful to miss I CJOEN for the expert drawing of the swimbladder I wish to acknowledge financial support from A/S Norsk Varekrigsforsikrings Fond

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The Role of the Cardiovascular Response in the Resistance to Asphyxia of Avian Divers

By

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Abstract

HOLLENBERG N K and B UVNAS *The role of the cardiovascular response in the resistance to asphyxia of avian divers* Acta physiol scand 1963 *58* 150—161 — Submersion asphyxia in unanaesthetized ducks produced a characteristic circulatory response consisting of a pronounced bradycardia increased blood pressure decreased splanchnic and skin blood flow and little change in skeletal muscle blood flow The circulatory responses to asphyxia were completely abolished by denervation of the carotid chemoreceptors and baroceptors Continuous measurement of arterial oxygen saturation showed a biphasic fall during asphyxia decreasing very rapidly during the first 40 sec and then falling much less rapidly at the time splanchnic and skin blood flow reached a minimum It was concluded that the marked ability of divers to withstand asphyxia is related to their circulatory response The net result of the circulatory response is to decrease the oxygen supply to areas that can easily withstand an oxygen deficit for a limited period conserving the available oxygen stores for tissues more sensitive to a lack of oxygen Evidence is presented which suggests that the above described response is due to chemoreceptor stimulation

Natural divers are able to withstand prolonged asphyxia because of their ability to decrease their oxygen consumption during submersion (SCHOLANDER 1940 ANDERSEN 1959) While a marked circulatory response has long been considered one of the major mechanisms by which divers conserved their oxygen stores (IRVING 1939) much of the evidence for changes in regional blood flow has been indirect According to IRVING s hypothesis decreased blood flow to those tissues which can operate anaerobically for a limited time

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such as skeletal muscle and intestine, spares the available oxygen stores for other tissues such as the brain and myocardium that do not tolerate an oxygen debt

In previous experiments when arterial haemoglobin oxygen saturations have been measured during submersion they have always been done on arterial blood samples removed from the animal generally only one sample being available during a dive In those cases where serial measurements were made, the samples were few in number and separated by a rather long time interval for technical reasons (SCHOLANDER 1940) The development of oximetry has made it possible to measure arterial oxygen saturation continuously (NILSSON 1960) so that data on moment to-moment changes could be obtained and correlated with concomitant changes in blood flow distribution to various tissues

Methods

Forty domestic ducks weighing 2 0 to 3 4 kg have been studied in all All ducks were blindfolded at the beginning of the experiments as this was found to calm them Skin incisions were made after local infiltration with 0 5 or 1 0 % Lidocaine© (xylocaine) It was found that, if tension on tissues was avoided by making long incisions and manipulation near peripheral nerves was avoided the procedures seemed to excite the ducks very little as evidenced both by their behaviour and early experiments in which heart rate and blood pressure were monitored during the more extensive procedures that followed

Preliminary experiments in which intraperitoneal sodium pentobarbital and intravenous chloralose or chloralose urethane were used all showed different results with a marked tachycardia and damping of the characteristic cardiovascular response to asphyxia

Arterial blood pressures were recorded from a brachial or sciatic artery by a pressure transducer (Statham P23 AA) Heart rate was determined either by an interval recorder via impulses from the pressure transducer or by increasing paper speed on the polygraph so that heart rate could be determined from the blood pressure recording Blood flow was recorded from the sciatic artery brachial artery or vein and superior mesenteric vein usually with the aid of a silicone filled drop chamber operating an ordinate recorder (LINDGREN 1958) When sciatic artery flow was measured the leg was skinned and a ligature tied tightly above the web isolating the web from the circulation In two pairs of ducks sciatic arterial flows were cross-circulated by way of drop chambers so that each duck had a leg perfused from the opposite duck

In order to measure blood flow to a predominantly skin preparation when brachial arterial flow was measured the drop chambers were inserted distal to the origin of the well developed proximal musculature and the greater part of the distal wing musculature was tied off

Carotid chemoreceptor and baroreceptor denervation was successfully accomplished in 3 ducks utilizing a posterior approach Since the operative procedure was too extensive to be done under local anaesthesia the ducks were anaesthetised with "Thiogenal© sodium methitural 15—20 mg/kg i v This is a short acting barbiturate without atropine like effects (GOODMAN and GILMAN 1956) The carotid bodies were identified low in the neck and the tissues surrounding them divided between ligatures since it was found technically impossible to selectively denervate the chemoreceptors

without opening the thorax and destroying the cervical air sacs As a result both the chemoreceptors and baroreceptors were denervated The carotid artery walls at the level were also infiltrated with 1 % xylocaine containing epinephrine The vagi were *preserved during these procedures The skin incisions were then closed with skin clips* and the ducks allowed to recover from the anaesthetic for 6 to 8 hours One duck was given a similar dose of Thiogenal and sham operated but its chemoreceptors were left intact and its responses had returned to normal within 3 hours of being anaesthetised

Continuous measurement of arterial oxygen saturation was accomplished utilizing the Atlas Doppel oximeter The procedure and apparatus used were exactly as described in detail by ROSELL and UVNAS (1962) The oximeter earpiece was fitted with a through flow cuvette connected in series with an arterial drop chamber through which blood from a sciatic artery was running The blood was returned to the duck by way of the distal portion of the sciatic artery Calibration was carried out using the duck's own blood Because of an often considerable drift in the instrument after calibration the earpiece was connected in series as rapidly as possible and only 3 to 5 submersions carried out before the oximeter was taken out of series and recalibrated The time interval between the initial calibration and recalibration was generally under half an hour

Submersion consisted of placing the duck's beak into a beaker of water so that the nostrils and mouth were held below water Care was taken to avoid any postural reflexes during this procedure Essentially similar responses were seen on manually asphyxiating ducks

When the drop chambers were used for measuring blood flow, heparin about 2: mg/kg was given intravenously Dextran (Macrodex© Pharmacia) was administered intravenously as required to compensate for blood loss except in those experiments where oximetry was done where blood loss was minimized by limiting the extent of the procedure as any lowering of hematocrit generally affected the results of oximetry (ROSELL and UVNAS 1962) Atropine 0.5 mg/kg was given intravenously to 10 ducks and 0 1 to 0 5 mg total dose intra arterially to another 12 ducks

Results

The results were obtained on over 400 periods of submersion in 40 ducks Blood pressure and heart were measured in all 40 ducks blood flow to leg wing or gut in 27 and oximetry in 9 Three ducks were studied after denervation of their cervical chemoreceptors and baroreceptors

Blood Pressure and Heart Rate

The average resting mean blood pressure in 40 ducks was 185 mm Hg (range 115—220) On submersion 38 out of 40 showed an average mean blood pressure increase of 32 mm Hg (range + 16 to +65) Two ducks showed consistent falls in blood pressure of 18 to 25 mm Hg during submersion Mean blood pressure increased from 115 mm Hg before submersion to a 165 mm Hg maximum at 60 sec in the example provided in Fig 1 The blood pressure increase seen during asphyxia was accentuated after intravenous atropine

All ducks developed a progressive bradycardia during submersion The fall in heart rate was generally apparent within seconds moderate within 15 sec

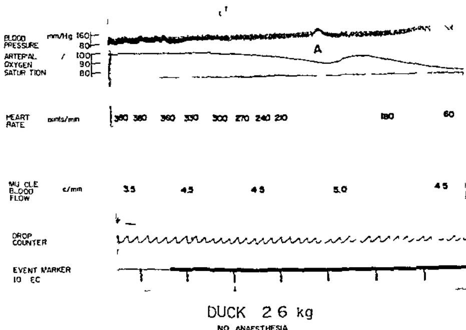


Fig 1 Blood pressure arterial oxygen saturation heart rate and muscle blood flow during asphyxia Note the very early fall in oxygen saturation and heart rate Tachycardia and increased arterial oxygen saturation at A was associated with a brief struggle
Duck — No anaesthesia

and progressed to a maximum fall by about 60 sec and was so maintained until the end of the period of submersion interrupted only by brief bursts of tachycardia during a struggle As an example the heart rate changes are clearly seen in Fig 1 where heart rate fell to 95 % of control within the first 5 sec was less than 70 % of control at 15 sec and had fallen to a minimum value of 16 % at 60 sec At A in Fig 1 a brief burst of tachycardia which was related to a period of struggling can be seen The minimum heart rates achieved during submersion were in the range of 15 to 35 % of control Afterwards a considerable tachycardia associated with hyperpnea was generally seen It is of interest that in the two ducks showing a consistent fall in blood pressure the bradycardia that developed during submersion differed in no way from the others *since it had been suggested that the bradycardia that many species* develop during asphyxia is due to baroceptor stimulation produced by an increase in blood pressure (KORNER and EDWARDS 1960) In all 10 ducks given atropine 0 5 mg/kg intravenously the early bradycardia was completely abolished but a less marked component that appeared after 60 sec was often still present The smaller doses of atropine given intra arterially had no apparent effect on either the heart rate or blood pressure response to asphyxia

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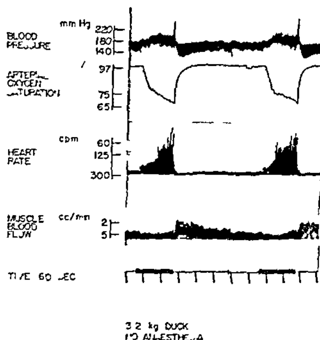


Fig 2 Blood pressure arterial oxyg n saturation heart rate and muscle bl od fl w changes during two successive periods of asphyxia Note the biphasic fall in arterial oxygen saturation with a marked retardation in the rate of fall at about 40 seconds
Duck — No anaesthesia

Blood Flow Measurements

Sciatic artery blood flow in the skinned leg with the web tied off was considered to present skeletal muscle blood flow In all 17 ducks in which it was measured fl w during the first 40 seconds of submersion (about the length of time of a normal dive ELIASSEN 1960) was equal to or very slightly greater than control flow levels In 11 of the ducks flow remained equal to control flow throughout the 2 to 3 min of asphyxia *e g* see Fig 2 The remaining 6 showed a minimal to moderate fall in flow after approximately 60 sec Intra arterial atropine in doses of 0 1 to 0 5 mg had no effect on muscle blood flow during submersion These doses had no apparent effect on the ducks bradycardia or pressor response to asphyxia

Prior to cross perfusion the changes in muscle blood flow during asphyxia described above were seen in all 4 ducks in which it was carried out However when the ducks legs were perfused by a resting donor muscle flow fell progressively despite a constant perfusion pressure indicating a neurogenically mediated increase in resistance in that bed as the nerve supply was the only remaining connection between the asphyxiated duck and the perfused limb As can be seen in Fig 4 A Duck N s muscle flow falls considerably during submersion

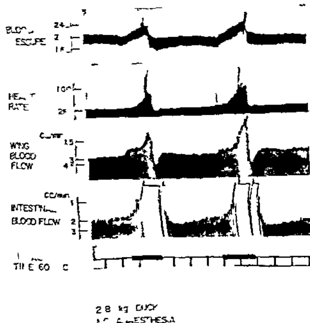


Fig 3 Blood pressure heart rate and skin and intestinal blood flo changes during two successive periods of asphyxia Note the marked fall in flow to intestine and skin as opposed to muscle blood flow in the preceding 2 figures.
Duck — No anaesthesia

despite the constant perfusion pressure supplied by Duck Y Also note Duck Y s increased muscle blood flow during Duck X s pressor response

Brachial arterial or venous flow was taken as an index of skin flow for reasons previously stated In spite of the increased blood pressure skin flow showed a progressive fall during submersion indicating a marked increase in resistance to flow The fall was generally moderate to marked and flow reached a minimum at approximately 40 sec (Fig 3)

Splanchnic blood flow in all ducks in which it was measured displayed a marked fall during submersion in spite of the increase in blood pressure again indicating a marked increase in resistance Flow also reached a minimum in 40 sec in this case and in one duck flow measured by a drop counter all but stopped completely after 40 sec until the end of submersion at 2 min (Fig 3)

Oximetry

Arterial oxygen saturation was measured in 9 ducks during submersion In spite of the precautions described under methods recalibration showed a 3 % or less change of baseline in only 4 ducks All the others showed changes of 10 % or more of baseline Qualitatively the results were similar in all 9 however

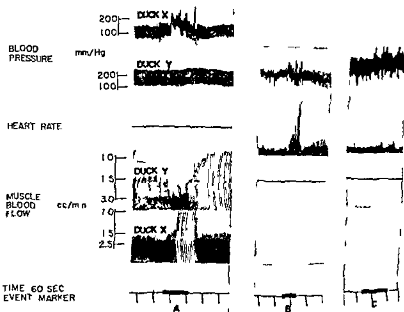


Fig 4 A) Blood pressures and cross perfused leg flow in 2 unanaesthetised ducks When Duck X asphyxiated note the decrease in Duck X s muscle blood flow perfused at constant pressure from Duck Y

B) Duck — no anaesthesia Typical pressor response with bradycardia during asphyxia

C) Same duck 8 hours later aft r denervation of carotid chemoreceptors and baroreceptors Note the increase in blood pressure and loss of the typical response to asphyxia

Thirteen submersions in the 4 ducks described above displayed the following changes in arterial oxygen saturation during submersion The fall in saturation was biphasic Saturation began to fall within 5 sec and fell rapidly for the first 40 seconds (mean 40 range 34—44) to a level of 80 % (mean 80 % range 77—82 %) The rate of decline then changed dramatically with a marked retardation in the rate of fall so that at 2 min the arterial saturation was still 70 % (mean 69 % range 62—78 %)

It should be noted that the time at which the decrease in arterial oxygen saturation during submersion showed a retardation in its rate of fall is the same as the time at which skin gut flow reached a minimum

Carotid Chemoreceptor and Baroreceptor Denervation

The acute effects of denervation included a steep increase in systolic and diastolic blood pressures in just under a minute with little change in heart rate as has been described in aneasthetised dogs (LEVY BRIND and BRANDLIN 1955) The increase in blood pressure gradually waned over the next hours so that when the ducks responses to asphyxia were again tested 6 to 8 hours

later their blood pressure had fallen somewhat but were still well above control. However, as can be seen in Fig. 4, B and C, at this time their characteristic circulatory response to asphyxia which in each case had been as previously described was abolished so that there was some tendency to increase in heart rate during asphyxia and the characteristic pressor response was gone. One of the ducks succumbed after only 2 to 3 min of asphyxia which is much less than they are usually able to tolerate. To rule out the possibility that the Thiogenal was still acting after this long time interval another duck was given an equivalent dose and sham operated and had normal responses within 3 hours.

Discussion

The circulatory response to asphyxia in divers and the mechanism by which it is produced seems to be clear. Progressive asphyxia acting by way of chemoreceptor stimulation produces an increase in peripheral resistance and an early and progressive fall in heart rate. Since the heart rate generally falls to one quarter to one sixth of control levels during asphyxia, it seems likely that there is a substantial associated fall in cardiac output, as it is unlikely that stroke volume could increase 4 to 6 times normal. This is further suggested by the findings of DOWNING, REMENSNYDER and MITCHELL (1962) in anaesthetised dogs that if respiration was controlled carotid body stimulation with hypoxic blood produced a fall in cardiac output with a bradycardia that was substantially less than those reported here. Blood pressure rose despite these factors because of a concomitant increase in peripheral resistance, especially in the splanchnic and skin vascular beds. The net effect of the fall in flow to various beds is that oxygen consumption is decreased. That the fall in oxygen consumption and circulatory responses are causally related is further suggested by the finding that the early, precipitous fall in arterial oxygen saturation becomes much more gradual at 40 sec, when the heart rate and tissue blood flows approach a minimum.

KORNER and EDWARDS found essentially similar responses to hypoxia in one group of unanaesthetised rabbits, *i. e.*, they responded to hypoxia with a progressive early bradycardia, fall in cardiac output, fall in oxygen consumption and a concomitant increase of blood pressure and total peripheral resistance (KORNER and EDWARDS 1960). These responses were abolished by chemoreceptor denervation. They suggested that the marked bradycardia and thus by inference the fall in cardiac output and the hypodynamic state was secondary to baroreceptor stimulation produced by the increased blood pressure due to intense peripheral vasoconstriction. However, NEIL has offered evidence that tachycardia associated with hypoxia is not due to chemoreceptor stimulation (NEIL 1956). Furthermore, the demonstration by DALY and SCOTT that lung denervation in dogs converts the tachycardia associated with hypoxia to a pronounced bradycardia suggests that the tachycardia usually seen with

hypoxia is secondary to hyperventilation the reflex effects of which mask the chemoreceptor response (DALY and SCOTT 1958) Since divers become apneic during asphyxia the tendency of chemoreceptor stimulation to produce bradycardia becomes manifest This is further suggested by our finding of bursts of tachycardia accompanying respiratory efforts *e g*, Fig 1 A and the two ducks in which asphyxia produced a fall of blood pressure associated with a pronounced bradycardia While KORNER and EDWARDS believed that the low output state was not advantageous in rabbits the advantage to divers of a mechanism for decreasing their oxygen consumption for a relatively short period is obvious

The marked fall in splanchnic and skin blood flow determined by direct measurement substantiates the suggestion that blood flow redistribution is an important factor in the ability of diving animals to tolerate prolonged asphyxia However, the finding that skeletal muscle blood flow failed to fall appreciably in the first two to three minutes of asphyxia when it would be important in the conservation of oxygen stores was unexpected IRVING demonstrated decreased muscle blood flow in many species during apnea using a thermocouple (IRVING 1938) However both SCHOLANDER (1940) and ANDERSEN (1959) found decreased body temperature related to the fall in metabolism during asphyxia so that muscle temperature would have fallen without a fall in blood flow Also SCHOLANDER (1940) found in ducks penguins and seals that blood lactic acid stayed close to normal during submersion and increased markedly afterward while muscle lactic acid increased during a dive and fell afterward He concluded from this that skeletal muscle blood flow was decreased during dive It seems likely that the prolonged periods of asphyxia he used six minutes and more would have led to a sufficiently severe degree of hypoxia as to lead to anaerobic glycolysis in skeletal muscle whether muscle blood flow changed or not

It is possible that muscle blood flow does fall after six minutes of asphyxia Six out of seventeen ducks showed some fall of muscle flow by the time two to three minutes of asphyxia had gone by However the results of oximetry indicate that the quantitatively important conservation of oxygen begins before this time in the first minute so that changes in total skeletal muscle blood flow do not seem to be critical in the resistance of the duck to asphyxia ELIASSEN was not able to demonstrate the above mentioned changes in lactic acid concentrations during rather shorter dives in ducks (ELIASSEN 1960) The possibility of shunting of blood flow away from nutritional vessels in the microcirculation of the skeletal muscle bed *e g* through the thoroughfare channels described in skeletal muscle by CHAMBERS and ZWEIFACH (1947) has not been ruled out in these experiments There is evidence that the sympathetic cholinergic vasodilator nerves to skeletal muscle produces functional shunting (ROSELL and UVNAS 1962 CHAMBERS and ZWEIFACH 1947) and the finding that their activation led to decreased oxygen consumption in muscle (ROSELL and UVNAS

1967) prompted UVNAS to suggest that they may be of importance in divers (UVNAS 1960) In the present experiments intra arterial atropine in substantial doses had no effect on skeletal muscle blood flow Furthermore the cross circulation experiments revealed that there was a neurogenically mediated tendency toward vasoconstriction in skeletal muscle which was masked in the autoperfused duck by some combination of the increased blood pressure hypoxia and hypercapnea However the vascular reflexes elicited by dipping the beak into water may not correspond to those during a dive when skeletal muscle activity concomitantly occurs

JOHANSEN and KROG measured the rate of venous pressure rise in a side branch of the femoral vein after the main venous return from the limb was occluded as an indirect index of arterial inflow to that bed (LEVY *et al* 1955) They interpreted their finding of a decrease in the degree of venous pressure rise on occlusion during submersion as evidence of decreased limb blood flow The difference between their conclusions and those reported here by direct measurement may be partially explained by the fact that they did not skin the limb or tie off the web and skin flow does fall during submersion It is also highly possible that there is a reflex venous dilatation associated with the fall in cardiac output and thus produced the fall in venous pressure Their finding that unobstructed venous pressure fell 10 mm Hg during submersion supports the hypothesis that what they were actually measuring was venous dilatation

The change in slope of the oxygen saturation fall during asphyxia could be explained either by a release of oxygenated red blood cells from the spleen or a decrease in utilization of oxygen STURKIE investigated the first alternative and found that ducks do not release red cells from the spleen during asphyxia (STURKIE 1943) so that decreased utilization is the probable explanation It is of interest that the decreased rate of fall occurred at 80 % haemoglobin saturation since this is precisely the point at which the opposite effect would have been expected from the shape of the oxyhaemoglobin dissociation curve which has a sigmoid shape as in other species Above 80 % saturation the fall in saturation relative to each increment of fall in oxygen tension is rather small while below 80 % the curve changes slope so that the fall in saturation increases markedly relative to the fall in tension Therefore it would have been expected that below 80 % saturation in the ducks the rate of fall would have been greater than rather than less than the rate fall above 80 % The decrease in the rate of fall attests to efficiency of the compensatory mechanism

This finding correlates well with those of ANDERSEN (1959) who found that while there was a marked fall in oxygen consumption during submersion asphyxia in ducks there might or might not be a change in the first minute

For technical reasons it was not possible to denervate the carotid chemoreceptors without denervating the baroreceptors at the same time since in the duck they lie very low in the neck actually within the scaphoid upper portion

of the thorax (Ask Upmark 1935), so that after identifying the structures from above it was only possible to denervate them by tying a series of ligatures around the tissue in the area preserving the major vessels and the vagi However since the baroreceptors have been shown to be insensitive to changes in oxygen tension carbon dioxide tension and pH (Euler Liljestrand and Zotterman 1941) it seems very likely that the abolition of the characteristic circulatory response to asphyxia after their denervation was due to loss of the chemoreceptors

Sturkie, while enumerating a long list of possible physiological roles for the air sacs in birds does not mention the possibility that they provide a store for oxygenated air available during submersion, or asphyxia of any sort (Sturkie 1954) While they have too sparse a blood supply for direct oxygen exchange (Sturkie 1954), the muscular movements made during diving would be *sufficient to force air from them into the lungs where exchange would take* place The finding that struggling produced moderate increases in arterial oxygen saturation (see Fig 1 A) is difficult to explain in any other way However this is not the only explanation for the decrease in rate of fall of oxygen saturation after 40 seconds since it occurred in birds that did not struggle at all

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Determination of the Hepatic Galactose Elimination Capacity after a Single Intravenous Injection in Man

The reproducibility and the influence of uneven distribution

By

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Abstract

TYGSTRUP N *Determination of the hepatic galactose elimination capacity after a single intravenous injection in man The reproducibility and the influence of uneven distribution* Acta physiol scand 1963 *58* 162—172 — The hepatic galactose elimination capacity was determined from the disappearance curve in blood after a single intravenous injection Using conventional principles for the calculation the results in repeated experiments were reproducible within 10 per cent Comparison of the results obtained by this method with those of an infusion method showed that the latter were consistently smaller This difference amounting to an average of 15 per cent might be caused by uneven distribution of galactose between its intra and extravascular volume of distribution From theoretical considerations it was deduced that the uneven distribution might be corrected for by parallel displacement of the arterial concentration curve along the time axis Experiments with continuous infusions at different rates indicated that on the average the curve of the mean concentrations in the body was delayed 7 min in relation to the arterial curve When the single injection experiments were corrected for this delay the difference between the results of single injection and infusion experiments disappeared

The elimination rate of galactose by the liver is assumed to be independent of the concentration in the blood at concentration levels which are easily obtained in clinical galactose tolerance tests (TYGSTRUP and WINKLER 1954 WALDSTEIN *et al* 1960) Under these conditions the galactose elimination rate may be of value as a liver function test

When the hepatic galactose elimination rate is calculated from the disappearance curve in the blood after a single intravenous injection the result may be influenced by the redistribution of galactose in the body and by the extrahepatic elimination The renal excretion of galactose can be corrected for approximately (TYGSTRUP 1961) The aim of the present work was to study the reproducibility and to assess the effect of uneven distribution by comparison of experiments with single injection and infusion of galactose

Methods

Experimental procedures

Most of the subjects studied had no evidence of liver disease but some patients with cirrhosis of the liver were included for comparison The experiments were performed in the morning while the subjects were still lying in their beds. They were kept fasting for 15 hours but were allowed to drink moderate amounts of water No premedication was given, Galactose (MERCK c. p) was injected intravenously in aqueous solution, sterilized by filtration.

In experiments with *single injection* 100 ml of the solution was injected intravenously at a constant rate in the course of 6 min. Twenty minutes after the injection blood samples were drawn from a brachial or femoral artery in subjects with normal liver function for 24 min at intervals of three minutes, and in patients with reduced elimination of galactose for 50 min at intervals of five minutes The urinary excretion of galactose was determined in a sample voided some hours after the end of the blood sampling period

In experiments with *continuous infusion* the solution was administered by calibrated, motor-driven syringes The volume infused was between 0.5 and 2.5 ml per minute the variation being less than 0.5 per cent. Arterial blood samples were drawn 20 to 30 min after beginning of the infusion, and at least 4 on the average 6 samples were taken at regular intervals during a period lasting for 20 to 60 min. With intervals of 15 to 30 min urine was collected through a bladder catheter

The concentrations of galactose in plasma urine and the solutions injected were determined as described by TYGSTRUP *et al* (1954) Plasma concentrations were converted to concentrations in plasma water by multiplication with the factor 1.05

Calculations

Only plasma samples in which the concentration of galactose exceeded 500 mg/l were used for the calculations since the hepatic galactose elimination rate usually falls at lower concentration levels (TYGSTRUP and WINKLER 1958) Only concentration time curves which appeared rectilinear were used and their slopes were calculated by regression

In the single injection experiments the hepatic galactose elimination capacity (*GE*) and volume of distribution (*V*) were calculated by the equations given by TYGSTRUP (1961)

$$GE = (M - U_{total}) / t_{=0} \quad (1)$$

$$V = M/c_{t=0} \quad (2)$$

where

M = the amount injected

U_{total} = the amount recovered in the urine

Table I Reproducibility of the results in repeated experiments with single injections of galactose (500 mg per kg body weight)

a Two successive determinations (A and B) on different days in 11 normal subjects and two patients with cirrhosis of the liver 10 males and 3 females age 16 to 66 years body weight 48 to 8? kg

		g (mg/l/min)	$c_{t=0}$ (mg/l)	$t_{c=0}$ (min)	V (l)	GE (mg/min)
Exper A	Mean	4? 0	2 470	61 0	13 8	50)
$\frac{A-B}{A}$ 100	Mean	−1°	−1	+ 1	+ 3	0
	S D	8 3	6 3	3 3	6 1	6 9

b Two successive determinations (A and B) on the same day in 5 normal subjects and two patients with cirrhosis of the liver 4 males and 3 females age 18 to 60 years body weight 52 to 69 kg The second injection was given 3? to 122 minutes after the first one the residual concentration (19 to 1 110 mg/l) was subtracted from $c_{t=0}$ of experiment B

		g (mg/l/min)	$c_{t=0}$ (mg/l)	$t_{c=0}$ (min)	V (l)	GE (mg/min)
Exper A	Mean	44 5	2 450	59 5	13 3	51?
$\frac{A-B}{A}$ 100	Mean	+ 5	+ 3%	−1	−3%	0
	S D	13 3	11 3	8 2	7 8	7 7

c Seven successive determinations on different days in the course of two weeks in one normal male, age 26 years, body weight 82 kg

	g (mg/l/min)	$c_{t=0}$ (mg/l)	$t_{c=0}$ (min)	V (l)	GE (mg/min)
Mean	44 0	2 520	6? 1	14 8	5?0
S D	7 44	336	3 53	1 83	4?
Coeff of variation	16 9	13 3°	5 7	1? 4 %	8.1

Explanation of symbols g = slope of the rectilinear part of the elimination curve in plasma. $c_{t=0}$ = the extrapolated concentration at zero time $t_{c=0}$ = the extrapolated time at zero concentration, V = the volume of distribution GE = the hepatic galactose elimination capacity

$t_{c=0}$ = the extrapolated time at zero concentration of the rectilinear part of the elimination curve and

$c_{t=0}$ = the extrapolated concentration at zero time

From these equations GF and I are calculated on the average three and two per cent too high owing to incomplete correction for urinary loss

Table II Comparison between single injections and infusions

	Age (years)	Body weight (kg)	Single injection			Infusion		
			−g (mg/l/min)	V (l)	GE (mg/min)	I—U (mg/min)	−g (mg/l/min)	f
J H	17	73	−48.5	14.9	644	954	+ 31.8	0.86
I R.	28	70	−55.2	13.5	64[illegible]	585	− 0.9	0.91
S C.	37	76	−50.5	13.1	590	603	+ 2.3	0.97
V G	47	65	−56.7	11.9	582	571	+ 4.4	0.90
B E.	27	68	−51.1	12.7	574	558	+ 6.2	0.8[illegible]
G N	42	55	−57.9	11.0	533	492	+ 9.5	0.77
A A.	39	65	−50.6	11.4	477	443	− 2.2	0.98
A L.	48	65	−30.2	15.6	430	300	− 9.0	1.04
						296	− 10.7	1.12
G P	38	49	−26.5	15.2	381	531	+ 21.1	0.79
M P	18	58	−36.6	11.0	354	265	+ 2.2	0.70
P R.*	42	86	−16.7	19.6	251	216	+ 0.4	0.84

Initials marked with designate patients with cirrhosis of the liver
For explanation of g V and GE see legend of Tab I
(Note The slope of a falling curve is considered to be positive)
I—U = infusion rate minus urinary excretion rate $f = (I-U)/(-g \cdot V_{singl} + GE_{singl})$

In infusion experiments with two infusions at different rates in the same subject, *GE* and *V* were calculated by

$$GE = \frac{g_B\,(I_A - U_A) - g_A\,(I_B - U_B)}{g_B - g_A} \quad (3)$$

$$V = \frac{(I_A - U_A) - (I_B - U_B)}{g_B - g_A} \quad (4)$$

where

g_A and g_B = the slopes of the arterial time-concentration curves in experiments *A* and *B* (the slope of a falling curve is defined as positive)
I_A and I_B = the infusion rates and
U_A and U_B = the mean urinary excretion rates in the intervals studied

Equations (3) and (4) are based on the series increment method of Lewis (1950) and imply that *GE* and V are identical during different infusion experiments in the same subject and that *I — U* can be regarded as a constant in the interval used for determination of the slope When more than two infusion experiments were performed in the same subject *GE* and V were calculated by regression according to the same principle

Results

In Table I are given the results of double determinations performed at an interval of one to 7 days (a) and on the same day (b) In (c) is shown the results from 7 determinations in one normal subject performed in the course of two weeks It appears that in all three series the variation of the hepatic galactose

Table III Comparison between single injections and infusions with different slopes

	Age (yrs)	Body weight (kg)	Single injection			Infusion			*Series increment	
			−g (mg/l/min)	V (l)	GE (mg/min)	I—U (mg/min)	−g (mg/l/min)	f	V (l)	GE (mg/min)
E J	25	91	−50.6	14.2	609	973 618 292	+37.0 +10.6 −18.4	0.86 0.81 0.84	12.3 (87%)	509 (84%)
H I	54	79	−44.9	14.1	596	683 −13	+16.1 −45.1	0.83 —	11.4 (81%)	499 (84%)
V C.	36	52	−44.7	13.6	563	753 −67	+37.3 −54.6	0.70	8.9 (65%)	470 (75%)
B A	19	70	−35.4	16.6	512	547 219	+7.0 −18.5	0.87 1.07	12.9 (78%)	456 (89%)
B F	43	45	−56.9	10.1	505	649 369 −83	+22.5 −9.5 −48.6	0.89 0.90 —	10.3 (102%)	435 (86%)
S J	23	62	−39.0	12.1	407	431 267 −56	+11.7 −9.5 −36.1	0.79 0.91 —	10.3 (85%)	330 (81%)
E P	30	67	−30.8	14.3	392	554 298	+15.9 −2.4	0.90 0.84	14.0 (98%)	337 (85%)
P N	44	59	−33.9	12.8	367	1 068 541 272 −59	+78.6 +40.3 −3.4 −37.0	0.78 0.61 0.84 —	9.7 (76%)	249 (68%)
K B	55	59	−31.7	12.9	341	374 186 −49	−8.2 −11.8 −31.5	0.84 0.93 —	10.7 (83%)	295 (87%)
M R.*	45	44	−23.6	13.5	302	426 245	+18.9 −3.0	0.77 0.94	8.3 (61%)	270 (89%)
K H*	50	75	−14.9	18.0	257	309 203	−9.8 −2.0	0.72 0.71	13.5 (75%)	177 (70%)

For explanation of symbols see legend of Tab. I and II

elimination capacity is about 10 per cent. The average difference between the double determinations in (a) and (b) is not statistically significant. In normal subjects and patients with cirrhosis of the liver there was no difference as to reproducibility; therefore they have been dealt with together in the tables.

The data from the experiments with single injections and infusions in the same subjects are given in Tables II and III, the latter including experiments with infusions at different rates.

In 6 experiments of Table III (characterized by negative values of I—U) the post infusion slope on the average was one per cent (S.E. 6 per cent) smaller than the slope following a single injection.

In 10 experiments (6 from Table II and 4 from Table III) an approximately constant arterial concentration, i. e. between −5 and +5 mg/l/min. was obtained by infusion. The slope was on the average +0.1 mg/l/min., the infusion rate minus urinary excretion rate 370 mg/min. and the hepatic galactose elimination capacity calculated from the single injection experiments 422 mg/min. Thus the true hepatic galactose elimination capacity is on the average about 88 per cent of the value calculated from a single injection experiment.

In subjects with two or more infusion experiments (Table III) the hepatic galactose elimination capacity calculated by the series increment method was found to be on the average 82 per cent of that obtained in the single injection experiments in the same patients. Similarly the volume of distribution was found to be 81 per cent.

This difference between the results of the single injection and the infusion experiments may be expressed by the factor $f_{GE} = GE_{infusion}/GE_{single\ inj}$ and $f_V = V_{infusion}/V_{single\ inj}$. The results obtained in Table III indicate that f_{GE} approximately equals f_V and a common factor f, can be calculated in each infusion experiment by

$$I - U = f(-gV_{single\ inj} + GE_{single\ inj}) \tag{5}$$

The factor f calculated from all the infusion experiments of Tables II and III (except those where I = 0 in which the experimental error will have too great influence) and the corresponding single injection experiments is on the average 0.86 (S.D. 0.11, S.E. 0.019). Thus the hepatic galactose elimination capacity and the volume of distribution determined in the infusion experiments is on the average 86 per cent of that calculated from the single injection experiments. This value is not significantly different from that obtained by the approximately constant concentration method or by the series increment method but significantly different from 100 per cent ($p < 0.001$).

Discussion

It is conceivable that the difference between the elimination rates calculated from single injection and infusion experiments may be related to uneven distribution of galactose. When galactose is injected into an eviscerated animal from which no removal takes place galactose will leave the intravascular compartment for about two hours (LEVINE *et al.* 1950). In the intact organism galactose moves from intra- to extravascular compartments during and for a short period following an intravenous injection. As the elimination by chemical transformation in the liver or by excretion in the kidneys takes place from the blood the amount stored in the extravascular compartment during the initial period must re-enter the blood stream.

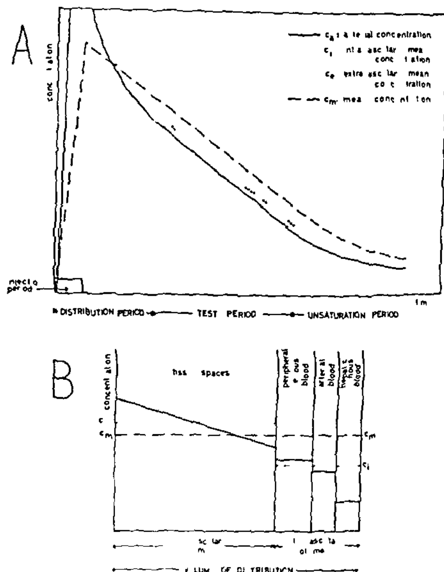


Fig 1 A hypothetical single injection experiment with the concentrat ons in d fferent compartments accord ng to the model consid red in the appendix A Time concentration curves B "Cross section" of A at a given time during the test period

Fig 1 shows a hypothetical distribution of galactose during a single injection experiment when it is assumed that the elimination rate and the volume of distribution are constant In this model (see appendix) uneven distribution results in parallel displacement of the arterial concentration curve relative to the curve of the mean concentrations in the system the horizontal distance

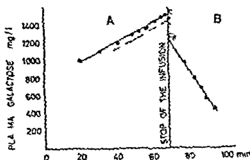


Fig 2 Plasma galactose concentration curves during (A) and following (B) a constant intravenous infusion The lines are drawn by rectilinear regression from the points situated at an approximately straight line and extrapolated to the point at the time axis where the infusion was stopped (c_A and c_B) The broken lines represent the theoretical mean concentrations in the volume of distribution They are drawn parallel to and the same horizontal distance from the respective regression lines and intersect at the moment the infusion was stopped

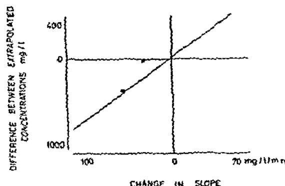


Fig 3 Relation of the difference between the extrapolated concentrations of a rising and a falling plasma galactose concentration curve and the difference in slope of these curves This ratio indicates the horizontal distance between the arterial and the theoretical mean concentration curves The average ratio calculated by rectilinear regression was 7 min

between the two curves being independent of their slope In infusion experiments the results calculated from the arterial curve (eq (3) and (4)) and the mean concentration curve will be identical The extrapolated values of the arterial curve ($c_{t=0}$ and $t_{c=0}$) used for the calculation in the single injection experiments (eq (1) and (2)) will be smaller than those of the mean concentration curve Thus single injection and infusion experiments will give different figures for the hepatic galactose elimination capacity and volume of distribution depending on the degree of displacement of the arterial curve

The displacement of the arterial concentration curves in the organism may be assessed by extrapolation of the arterial curves in experiments in which the rate of infusion is changed An experiment of this type is shown in Fig 2 The displacement is calculated as the ratio of the difference between the slopes of the rectilinear part of the curves or

$$k = (c_B - c_A) / (g_A - g_B)$$

(see appendix)

In 13 of the experiments shown in Table III the displacement could be calculated in this way Fig 3 depicts the relation between the data entering

As in Fig 2 $c_{i,A} \neq c_{i,B}$ At t_i the amount present in the model is $c_m I$, thus $c_{m,A} = c_{m,B}$ From eq (6) (7) and (8) the mean concentration curves in period A and B are described by

$$c_{m,A} = g_A (t - t_i - K) + c_{i,A} \quad (9)$$

$$c_{m,B} = g_B (t - t_i - K) + c_{i,B} \quad (10)$$

From eq (9) and (10) follows that

$$I = (c_{i,B} - c_{i,A})/(g_A - g_B) \quad (11)$$

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Factors Determining the Circulatory Adjustments to Diving

I Water immersion

By

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Abstract

ANDERSEN H T *Factors determining the circulatory adjustments to diving I Water immersion* Acta physiol scand 1963 *58* 173—185 — The importance of water immersion *per se* for the circulatory adjustments to diving has been studied in the domestic duck. The heart rate has been used as an index of the cardio-vascular changes The physiological reactions characteristic of diving were elicited upon submersion in ducks with free access to air through tracheal cannulas just as well as in intact birds Likewise the adjustments to diving were maintained by emerging ducks after the nostrils were above the water surface provided the rest of the beak was kept submerged Also when the lungs and air sacs were ventilated at the normal rate during descent and submersion the diving bradycardia was nevertheless elicited By studying the heart rate during slow submersions of the beak and the head it was found that the most marked cardiac slowing resulted from immersion of the level of the nares whereas the conspicuous post-dive tachycardia was brought about during the first respiratory effort It is concluded that the circulatory adjustments to diving are elicited by the actual water immersion probably due to stimulation of peripheral receptors in the beak especially in the region of the nostrils The degree of distention of the lungs and the air sacs as well as variations in the venous return may also be important factors for the elicitation and abolition of the diving characteristics

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Experimental water immersion is associated with a characteristic series of respiratory and cardio vascular responses in diving animals Apnoea is induced immediately upon descent and persists throughout the period of submergence A conspicuous bradycardia develops and the flow and pressure relations in the vascular system are greatly modified The circulating blood is probably channelled in such a way that the central nervous system and presumably the heart receive an adequate supply of oxygen whereas tissues not so easily damaged by transient but severe asphyxiation are left without access to oxygen As a result, the energy metabolism is depressed during a prolonged, quiet submersion, and the homoiothermic divers suffer a corresponding fall in body temperature All these reactions have been observed in a number of diving species among the reptiles birds and mammals (BERT 1870 RICHET 1899 HUXLEY 1913 a c IRVING *et al* 1935 IRVING 1938 SCHOLANDER 1940, SCHOLANDER and IRVING 1941 IRVING SCHOLANDER and GRINNELL 1941 1942 GRINNELL IRVING and SCHOLANDER 1942, SCHOLANDER, IRVING and GRINNELL 1942 a b ANDERSEN 1959 a, b 1961, JOHANSEN 1959 JOHANSEN and KROG 1959, ELIASSEN 1960, MURDAUGH *et al* 1961, MURDAUGH and JACKSON 1962)

It seems reasonable to assume that the physiological adjustments to diving are elicited either by the asphyxiation which progressively increases during a prolonged submersion or by the actual water immersion Already BEAU (1860), believed that the apnoeic response to water immersion seen in dogs is a result of the submergence *per se* whereas BERT (1870) ascribed this reaction to voluntary breath holding HUXLEY (1913 a) showed that apnoea is induced in decerebrated ducks upon submersion just as well as in intact individuals thus proving that the cessation of breathing observed does not depend upon higher levels of the central nervous system

As for the circulatory responses SCHOLANDER (1940) found in seals well accustomed to the experimental procedure that the diving bradycardia was elicited and maximally developed momentarily upon descent ELIASSEN (1960) claimed that the bradycardia is brought about very rapidly also in certain avian divers under natural conditions An immediate and precipitous drop in the heart rate would certainly indicate that the cardio-vascular adjustments to diving like the apnoea are reflexly initiated in response to the submergence However such clear cut changes in the circulatory functions are rarely seen for even when a definite decrease in the heart rate takes place in the beginning of a dive the cardiac frequency may continue to slow gradually for a relatively long period of time before the final level of bradycardia is established Furthermore, in the majority of the animals studied less well trained seals included (IRVING *et al* 1941) such a gradual cardiac retardation is the rule (SCHOLANDER 1940, SCHOLANDER and IRVING 1941 JOHANSEN 1959 ANDERSEN 1961, MURDAUGH and JACKSON 1962) Now it has been shown that rebreathing of air as well as high carbon dioxide content in the inspired air causes cardiac slowing in seals and semi aquatic snakes (IRVING *et al* 1935 SCHOLANDER 1940 Jo-

HANSEN 1959) and apnoea in the duck (ORR and WATSON 1913) In addition SCHOLANDER (1940) found that when seals are given pure nitrogen to breathe bradycardia and irregular respiration or complete apnoea results Likewise the normal post diving tachycardia is rapidly replaced by bradycardia and apnoea if seals are subjected to a nitrogen atmosphere in the recovery period Obviously the possibility remains that the cardio-vascular adjustments to under water exposure may be determined by the asphyxia incurred or by one of its two components hypoxia or hypercapnia respectively

The purpose of the present investigation, reported in this and a subsequent paper has been to separate the effects of asphyxia and water immersion *per se* as far as possible in order to evaluate the relative importance of these two factors for the circulatory responses to diving The heart rate has been used as an index for the appearance and development of the cardio-vascular changes In this paper the significance of the actual water immersion is dealt with

Material and Methods

Animals and animal care

Thirty-six domestic ducks of both sexes 1—2 years old served as subjects in the present investigation Their body weights ranged from 2.5 to 3 8 kg when they first arrived at the institute They were fed boiled potatoes bread, grain and various vita minized provenders *ad lib* and had unlimited access to running water As a rule the animals gained weight during their stay at the laboratory none of them loosing weight Thus the subjects presumably remained in good condition throughout the experimen tal period.

Arrangement of the diving experiments

During the experiments the ducks were fastened to a board as illustrated in an earlier publication (ANDERSEN 1959 a) and the fact that all the diving characteristics are elicited when only the head of the animal is immersed into water (ANDERSEN 1959 a, b 1961) was made use of also in the present investigation Conditions of diving were therefore simulated either by gently tilting the board to a slight angle into a water pool or by placing a mask subsequently to be filled with water over the beak and the head of the duck. The mask was made from a plexi glas cylinder which was closed in one end and fitted with a rubber sleeve in the other The rubber sleeve could be pulled back over the head of the duck in order to provide a water tight seal around the neck and the cylin der could be filled with water through large pipes near the closed end In periods of rest or recovery the duck could breathe air through these pipes

In a series of experiments the lungs and air sacs of the ducks were ventilated arti ficially For this purpose tracheal cannulas were inserted under local anesthezia provided by infiltration with a 2 % solution of xylocain.

The temperature of the water into which the head of the animal was submerged was varied between 15 and 30 C in order to learn whether this would alter the respon ses to diving In no experiment however was the temperature of the water found to influence the physiological adjustments to diving

Heart rate as indicator of circulatory changes

The various physiological adjustments to diving are always elicited in response to water immersion of the head Furthermore the changes with time of the individual

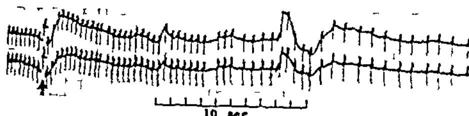


Fig 1 Gradual development of diving bradycardia in duck Head submerged at arrow

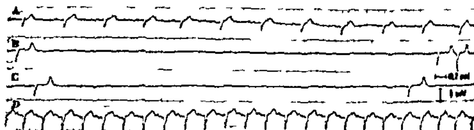
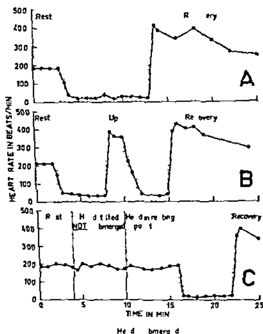


Fig 2 ECG tracings (lead III) from duck

A Before submersion
B Head submerged 1 min
C Head submerged 9 min
D Immediately after emergence

parameters differ very little Therefore these functional modifications are neither separately induced nor independently developed

Considering the cardio-vascular reactions in particular it appears obvious that the conspicuous diving bradycardia can not be an isolated circulatory modification but must be associated with equally marked changes in the flow and pressure relations in the cardio-vascular system Certain changes of this sort like for instance marked peripheral vasoconstriction and decreased or suspended circulation through skin muscles kidneys and viscera have been observed in all animal species studied which exhibit a diving bradycardia (SCHOLANDER 1940 IRVING *et al* 1941 GRINNELL *et al* 1942 JOHANSEN and KROG 1959 ELIASSEN 1960 ANDERSEN 1961 MURDAUGH *et al* 1961) Since the intimate relationship between the various cardio-vascular adjustments to diving thus has been demonstrated in a large material including representatives for mammalian avian and reptilian divers it was decided to use the heart rate which may be easily recorded with a minimum of discomfort to the animal as an index for the elicitation maintenance and abolition of the entire complex of cardio-vascular responses to submergence For this purpose needle electrodes were inserted subcutaneously into the thighs and at the base of the wings Electrocardiograms (ECG) were recorded using an Elema Schönander Mingograf Model 42 Because the heart rate is the factor of primary interest in the present investigation only one lead of the ECG is shown in the illustrations in this paper In order to record the ECG and the respiratory rate simultaneously one of the channels of the Mingograf had its DC-input connected to a condenser manometer which was coupled to an air filled tube strapped around the chest of the animal



F g 3 A H art rate before during and after water immersion of head for 10 min
B Heart rate in connection with e peated d ves (See text)
C Test for postural influence on heart rate (See text)

Results

Normal responses

The heart rate was always found to slow down gradually during the initial stage o a dive (Fig 1) A constant low cardiac frequency was usually estab lished in about 1 min of submergence at a level of 5—15 % o the pre diving heart rate and upon emergence a pronounced tachycardia was elicited (Fig 2)

The individual differences observed in the rate of cardiac slowing and in the final level of the diving bradycardia were relatively small A lower heart rate than 10 beats/min was very rare and the upper limit for the post-diving tachycardia appeared to be 450 beats/min The pre diving heart rate was usually restored in 15—20 min Fig 3 A shows a typical example of the changes in cardiac rhythm associated with the endurance of diving conditions for 10 min and in the subsequent period of recovery

The heart rate prior to diving was found to vary in the different individuals between 180—240 beats/min It appeared desirable therefore to determine to what extent the initial heart rate would influence the rate of development and the final level of the diving bradycardia This was done by subjecting some of the ducks to two successive submersions In this way the heart rate prior to the second water immersion would be almost twice the resting An example of the results obtained is furnished in Fig 3 B The heart rate before the first dive was 210 beats/min which decreased to 30 beats/min during the submergence

which totalled 6 min After an incomplete recovery period lasting for only 2 min the head of the duck was submerged for a second time the heart rate at this point being 360 beats/min During this second dive the heart rate slowed down to the same level as that observed in the first but the development of this level of bradycardia took twice as long

Postural apnoea and bradycardia

When a duck is placed on its back with the neck extended a so called postural apnoea accompanied by cardiac slowing may be induced This phenomenon has been explained to be due to vestibular functions (HUXLEY 1913 b c)

The ducks used in the present investigation were fastened ventral side down to a board but with the neck and head extended and tilted to an angle with the horizontal plane in order to immerse the head into the water pool Therefore it was felt necessary to make certain that the bradycardia observed during submergence was entirely due to the under water exposure This point was experimentally checked as follows The head of the animal was tilted down into the empty tub in order to remain in the position of the actual diving experiments for several min and subsequently returned to the resting position for a period of recovery During the latter the tub was filled with water and the control experiment performed The results obtained in one of these experiments are shown in Fig 3 C As is seen from the graph the tilting of the animal did not induce any significant change in the heart rate whereas in the control experiment performed 6 min later in which the head of the duck was actually submerged the normal response to diving was displayed

Peripheral stimulation due to submergence

The importance of peripheral stimulation due to water immersion of the head have been revealed in three different types of experiments A representative sample of the data obtained is shown in Fig 4 A B and C

The experiment illustrated in Fig 4 A was carried out on a duck fitted with a tracheal cannula The cannula was almost as wide as the trachea itself and its length had been adjusted so as to keep the respiratory dead space very close to normal The animal had been breathing through the tracheal cannula for about an hour when the experiment was performed The heart rate was 190 beats min prior to submergence As soon as the head of the duck became immersed into water apnoea was brought about and the diving bradycardia developed in the normal manner although the animal could have continued to breathe through its tracheal cannula which extended out of water After 4 min of submersion the bird was lifted out of the water pool and exhibited the usual post-diving tachycardia Eight min later the same experiment was performed but the tracheal cannula was clamped in the moment of descent Again the typical response to diving was observed followed by an equally typical recovery upon emergence

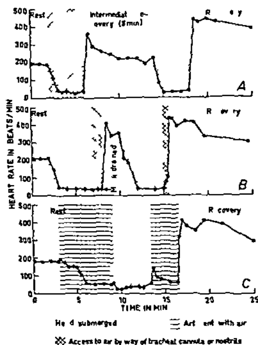


Fig 4 Heart rate connection with submersions in which the duck had access to air

A. Access to air through tracheal cannula
B Access to air by way of nostrils
C Lungs artificially ventilated
See text for details

In the experiment illustrated in Fig 4 B the head of the duck was introduced into a plastic cylinder which was filled with water A normal development of the diving characteristics was induced as seen from the graph After having endured this sort of diving conditions for 5 min the cylinder was drained until the nostrils were free above the water surface while the lower parts of the beak were still submerged Also some water may have been present in the nasal cavity In this situation no respiratory effort was attempted by the animal During the next 60 sec full diving conditions were maintained by the bird in spite of it having free access to the surrounding atmosphere Upon rapid and complete drainage of the cylinder at this point the forceful expiratory blow and subsequent deep inhalation typical of the first respiratory cycle after submergence was performed and abrupt tachycardia was elicited Two min later when the cardiac frequency still was 360 beats/min the experiment was repeated As before the diving bradycardia developed gradually to a level of about 30 beats/min When the cylinder was partly drained to the same level as in the first experiment a couple of feeble and half hearted respiratory cycles were performed during the following 30 sec but nothing like the forceful respiration which normally initiates the recovery period was observed The heart rate increased to 110 beats/min in response to this weak respiratory effort or to less than one third of the heart rate prior to the submersion When the cylinder was completely emptied cardio-acceleration to 440 beats/min took place in 15 sec

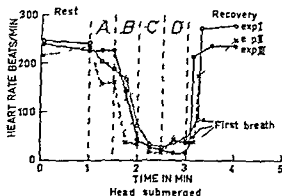


Fig 5 Receptive fields of beak causing diving bradycardia when immersed into water

A Beak rostral to nares submerged
B Nostrils and caudal part of beak submerged
C Whole head under water
D Eyes but no part of the beak above water

During the initial part of the Recovery the nostrils were above water but respiration was not started immediately Arrows indicate first breath

A further step along this line of experimentation was taken in the experiments represented by Fig 4 C A duck was fitted with a tracheal cannula and the latter connected to an apparatus for artificial ventilation The respiratory pump was set to deliver a tidal volume of 30—35 ml (SOUM 1896 VOS 1934 SCHARNKE 1938 quoted by STURKIE 1954) and the respiratory rate was adjusted as close as possible to that of the subject The respiratory pump was switched on in the resting period so that the artificial ventilation was synchronized with the respiratory cycles of the duck. The starting of the pump had a certain but relatively small effect on the heart rate which showed a transient increase followed by a slowing down to 150 beats/min or to roughly 85 % of the previous rate The cardiac rhythm remained constant at this new rate for 1 min before the head of the duck was submerged In spite of continued ventilation of the lungs and air-sacs at the pre-diving rate and tidal volume the heart rate slowed down rapidly in the usual response to diving conditions The bradycardia did however not slow down to quite the same low level as that seen in most control experiments but remained constant at a frequency of 60 beats min for 4 min When artificial ventilation was discontinued at this point a further slowing down in the heart rate was observed and a diving bradycardia of around 30—35 beats min was established After 4 5 min the respiratory pump was started again This caused a rapid but transient cardio-acceleration to 120 beats/min which was subsequently followed by a slowing in the heart rate until the level of bradycardia observed during the first part of the dive had become re-established When surfacing was allowed 3 min later, the animal was disconnected from the respiratory apparatus and a normal recovery ensued

Receptive field for stimulation by water immersion

It was assumed that by gradually and slowly submerging the head starting at the tip of the beak and proceeding caudally one might be able to determine the exact level of water immersion necessary to induce the physiological adj

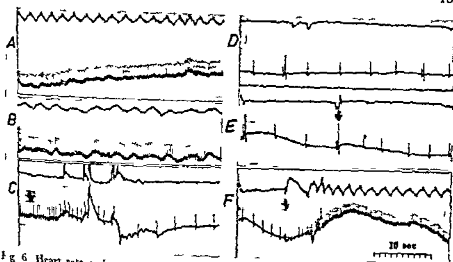


Fig 6 Heart rate and respiration recorded simultaneously before water immersion during descent submergence ascent and after surfacing Upper tracing respiration inspiration upwards lower tracing ECG
A Before submergence
B Beak rostral to nares immersed
C Nostrils and caudal part of beak under water (arrow)
D Whole head submerged
E Eyes but no part of beak above water (arrow)
F Nostrils above water first breath indicated by arrow Note the conspicuous slowing down in cardiac rhythm after the first inspiration

ments to diving By reversing the procedure one ought to be able to establish at which level of emersion of the head the diving characteristics are abolished A series of experiments were performed on this assumption and an example of three of them performed on the same subject within one half hour is shown in Fig 5 In the first and the second experiment the heart rate started slowing down as soon as the rostral end of the beak made contact with the surface of the water The rate of cardiac slowing decreased (experiment I) or stopped completely (experiment II) as long as the water level did not reach the nares (A) The main drop in cardiac rhythm occurred when the nostrils and subsequently the whole beak was immersed into water (B) and the diving bradycardia was finally somewhat extra accentuated when the whole head became submerged (C) The heart rate rose slightly when the head emerged so that only the beak remained under water (D) and when the nostrils were brought up above the water surface the heart rate increased further However only upon the first breath was the post diving tachycardia observed The result of the third experiment followed the general pattern of the first two but with two interesting differences namely no appreciable cardiac slowing took place before the nares became submerged and cardio-acceleration did not occur until the nostrils were free above the surface of the water again

In order to illustrate the simultaneous changes in heart rate and respiratory rate and amplitude the recordings obtained in an experiment are presented in Fig 6 The immersion of the head into water was carried out very slowly as described for the preceding experiments The upper tracing is the respiration (inspiration upwards), the lower a monopolar ECG

Fig 6 A shows the resting cardiac and respiratory rates to be 235 and 28 cycles/min, respectively Already when the very tip of the beak was dipped into water, both of these parameters became modified (Fig 6 B) The tidal volume became smaller and more variable and the respiratory rate decreased to 18 cycles/min The heart rate slowed down to 198 beats/min When the water reached the rostral border of the nares the respiration had become still more slow and shallow, and the heart rate had decreased to 156 beats/min (Fig 6 C left) Shortly after the nares were submerged the diving bradycardia was marked (Fig 6 C at arrow), and the heart rate decreased further when the whole head was submerged (Fig 6 D) The head remained fully immersed in water for 30 sec, when it was slowly pulled out again When the level of the eyes emerged the heart rate increased slightly (Fig 6 E at arrow), and quite markedly when the nostrils were lifted above water (Fig 6 F left) However only when the first, vigorous respiratory cycle was performed did the conspicuous post diving cardio acceleration take place (Fig 6 F at arrow) The first respiratory cycle usually consists of a forceful exhalation which apparently serves to blow the remaining water away from the respiratory orifices and the glottis Then follows a deep and fast inspiration, and a respiratory pause in the inspiratory position The latter may last for several sec, in the experiment shown in Fig 6 F it lasted 3 5 sec before a new forceful expiration took place During such respiratory pauses the heart rate was always found to slow down but it accelerated again when the respiration continued After only a few vigorous respiratory cycles the respiratory amplitude and rate returns to normal when the dive as in Fig 6 is relatively short

Discussion

Huxley (1913 a, c) proved that the apnoeic response to submersion is reflexly initiated by demonstrating that it takes place in the decerebrated duck as well as in the intact bird upon water immersion Whereas apnoea of course is induced immediately upon descent, the circulatory adjustments to submergence develop gradually during the initial part of a dive (Fig 1) Therefore one may question whether the cardio-vascular modifications during diving are produced by the water immersion *per se* or whether they are caused by asphyxiation The results reported in this paper give certain indications as to the importance of the actual submergence for the production of the circulatory characteristics of diving

First, it has been shown that an intact duck with free access to air through a tracheal cannula exhibits the apnoea and diving bradycardia in response to

immersion of the head into water (Fig 4 A) Next, it was found that when the water level was lowered so that the nostrils emerged after the duck had endured diving conditions for 5 min the animal would refuse or make only very feeble attempts to breathe provided the lower parts of the beak were still under water (Fig 4 B) Finally it has been demonstrated that even when the normal ventilation of the lungs and air sacs is maintained uninterrupted during descent and submergence by artificial means the diving bradycardia is nevertheless induced (Fig 4 C) These experiments strongly suggest that there are certain receptors in the region of the beak which are activated by submergence and that the nervous information from these peripheral receptors produces cessation of breathing and marked modifications in the circulatory functions The latter changes may be induced directly or as a result of the apnoeic response Furthermore the receptive field stimulated by water immersion appears to be the whole beak and possibly also the rostral part of the head the region of the eyes included However the most important zone by far for the elicitation of the physiological adjustments to diving seems to be the caudal part of the beak measured from the rostral border of the nares (Fig 5) This finding is supported by the observation of MURDAUGH and JACKSON (1962) that water snakes in which the nares were closed up did not display the adjustments to diving upon submergence SCHOLANDER *et al* (1962) express the opinion that the diving bradycardia is not a response to wetting of the nostrils They base their opinion upon the occurrence of bradycardia in the seal and the alligator induced by strong auditory stimuli or by frightening the animals in other ways (SCHOLANDER 1940 ANDERSEN 1961) Transient bradycardia is however induced by a variety of stimuli terrifying ones among others But in the case of diving wetting of certain receptors in the region of the respiratory orifices is almost certainly the adequate stimulus for eliciting the circulatory changes characteristic of diving

The water immersion although important does not appear to be the only factor determining the cardio-vascular reactions for when artificial ventilation was discontinued during submergence the heart rate slowed down further (Fig 4 C) Likewise upon re starting of the respiratory pump the cardiac rhythm was adjusted to the rate observed during the initial part of the dive There is therefore reason to believe that asphyxiation or the absence of rhythmic stretching of the lungs and air sacs may further accentuate the circulatory adjustments

The relationship between heart rate and respiration deserves some further comments IRVING *et al* (1941) found that cardio acceleration may take place in the seal upon emersion before the onset of respiration JOHANSEN (1959) however working on a semi aquatic snake never observed the post-diving tachycardia to precede the first inspiration The results of the present investigation seem to bridge this apparent difference of opinion For it has been shown that cardio-acceleration indeed takes place during emersion before the respira-

tion is resumed, whereas the conspicuous increase in heart rate which is usually seen at the beginning of the recovery period, occurs only with the first respiratory effort (Fig 6 E, F) The comparatively modest increase in heart rate during the slow emergence of the head and the beak may be due to the gradual decrease in the nervous influence from peripheral receptors The precipitous tachycardia displayed at the onset of the respiration on the other hand, is probably caused not only by the abolition of such nervous activity, but may also result from the respiratory *effort itself* The changes in the asphyxic condition appear less important in this connection since the cardiac response takes place so rapidly that the asphyxia can hardly have been relieved

The effect of the respiratory activity on the heart rate may be thought accomplished in two ways First, it is known that an increased venous return may cause a marked cardio acceleration especially when the heart rate is slow, either by stimulation of vagal afferents (BAINBRIDGE reflex) or by a direct action on the pacemaker cells of the heart JOHANSEN, KROG and REITE 1962) Second, the impulse traffic in vagal afferents resulting from the distention of the lungs and air sacs (HERING BREUER reflex) may influence the heart rate This is, for instance known to be one of the causes of sinus arrythmia, a phenomenon which is conspicuously exhibited by the emerging duck during the first respiratory cycle (Fig 6 F) Here it is of interest to relate the observation by SCHOLANDER (1940) that seals always dive on expiration and ELIASSEN s finding (1960) that avian divers which did not expel air upon submergence tended to developed the diving bradycardia very slowly The exhaling of air just prior to or upon submersion has been thought of as a protection against *caisson disease* or an adjustment of the buoyancy in deep diving However, a third interpretation of this phenomenon may be suggested, namely, that it also serves to facilitate the onset of the diving bradycardia and the other circulatory changes seen during water immersion

From the data presented in this paper it is concluded that the cardio-vascular adjustments to diving are induced by the submersion *per se* due to the stimulation of peripheral receptors located on the beak especially in and around the nares. The elicitation and abolition of the diving bradycardia may also be influenced by the degree of distention of the lungs and air sacs and by changes in the venous return

Asphyxia when added to water immersion appears to accentuate the diving bradycardia and the effect of asphyxic conditions will be dealt with in a subsequent paper

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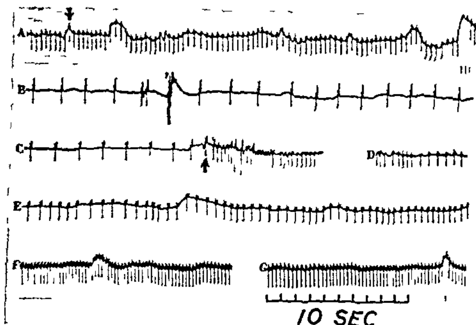


Fig 2 ECG-tracings recorded before during and after 4 min of tracheal occlu on

A Rest (left) Trachea occluded at arrow
B Trachea occluded 1 min
C Trachea occluded 4 min (left) tracheal clamp released at arrow
D Thirty sec after release of tracheal clamp
E One min after release of tracheal clamp
F Five min after release of tracl eal clamp
G Seven min after release of tracl eal clamp

Material and Methods

The present investigation was carried out simultaneously with a study of the s gn ficance of water immersion *per se* for the elicitation of the diving characteristics (ANDER SEN 1963) Therefore the same ducks were used and the methods employed were also identical except in the experiments in which the lungs and the air sacs were artificially ventilated during diving These differences are described below

The lungs and the air-sacs were ventilated artificially with air during submergence in order to relieve the asphyxia and determine whether or not the diving bradycardia could be abolished or modified by such a procedure Artificial ventilation was also performed using nitrogen so that the effects obtained with air could be compared to those produced by a respiratory inert gas Both during ventilation with air and ni rogen carbon dioxide is washed out of the organism but whereas the content of oxyg n tends to be restored to normal when air is used it will d crease gradually when nitrogen is employed *Therefore in order to study the separate effects of hypoxia and hypercapnia*, the lungs and air-sacs have to be ventilated with proper gas mixtures at an advanced stage of the dive when the asphyxia has become significant and the concentra ons of the gas components in the respiratory organs are not rapidly changing This was ac complished by making use of the analytical data on the respiratory gases *obtained* in previous studies (ANDERSEN 1959 a, b) *by means of which the gas composition* in the

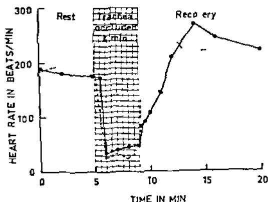


Fig 3 Graph showing the response in heart rate to 4 min of tracheal occlusion Note the initial delay before bradycardia develop and also the slow recovery of normal heart rate Dotted line Response in cardiac rhythm to 4 min of diving in the same animal

lungs of the duck could be reasonably well predicted at any time during submergence (Fig 1) For the purpose of the present investigation it was decided to start artificial ventilation of the lungs and air sacs after 5 min of diving using two gas mixtures one containing 20 % O_2 7 % CO_2 and 73 % N_2 the other containing 7 % O_2, 93 % N_2 and no CO_2 In this way it was attempted to relieve the hypoxia or the hypercapnia selectively Three different volumes of tidal gas were used in all of the experiments 25—30 ml which is somewhat less than the normal tidal volume of an adult duck in the standing position 50 ml and 100 ml. The latter tidal volume although seemingly large is not more than about 35 % of the total capacity of the lungs and air sacs (SUOMI 1896 VOS 1934 SCHARNKE 1938 quoted by STURKIE 1954)

However artificial ventilation of the respiratory organs during diving does not only modify the asphyxic condition but it also causes rhythmic stretching of the lungs and air sacs Since *this factor may be of importance for the abolition of the diving characteristics* (ANDERSEN 1963) it was decided to investigate the effect of the stretching *per se* This was done by carrying out artificial ventilation of the lungs and air sacs after 4—5 min of diving using a gas mixture containing 7 % O_2 7 % CO_2 and 86 % N_2 Hereby the composition of the gas in the respiratory organs was presumably not altered appreciably and only the mechanical stretching of the lungs and air sacs was varied by changing the tidal volume as described above

The heart rate before during and after water immersion was recorded in all of the animals used In this way the normal responses to submergence of each individual were available for comparison with those obtained when the lungs were artificially ventilated during diving

The effects of asphyxia alone was studied by occluding the tracheal cannula for a certain period of time

Results

Effects of asphyxia

The effects of asphyxiation alone on the heart rate and the respiration are shown in Figs 2 3 4 and 5 (first part) Although these illustrations may appear fairly similar to those representing diving conditions (ANDERSEN 1963) several important differences are present For instance during asphyxiation the heart rate would remain almost unchanged for up to 45 sec before a bradycardia was

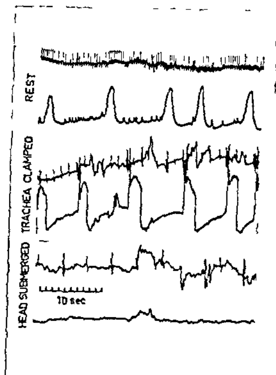


Fig 4 Comparison of respiratory activity and heart rate at rest during tracheal occlusion and submergence of head Note Complete apnoea during diving as opposed to rhythmic respiratory efforts at resting rate during tracheal occlusion All records show ECG (top) and respiration (bottom) Inspiration upwards

induced (Figs 2 3 and 5) and the latter was never quite as pronounced as that observed during diving (Fig 3) Upon release of the tracheal occlusion marked cardio acceleration would sometimes occur *(Fig 2 C) However after* a few sec the heart rate invariably decreased again to a comparatively slow frequency from which a gradual recovery took place (Fig 2 C—G) *Frequently*, no abrupt cardio acceleration was seen at all upon unclamping of the trachea, *but only the slow and gradual* one (Fig 3) *The most striking difference noted* during asphyxiation as compared with diving was however the respiratory behaviour of the animal For whereas immersion of the head into water causes complete apnoea which lasts throughout the diving period the rhythmic respiratory effort of the animal never ceased during tracheal occlusion *This is* illustrated in Fig 4

Summation of asphyxia and submergence

In a previous paper (ANDERSEN 1963) it was reported that the diving bradycardia was induced upon submersion of ducks whose lungs and air sacs were ventilated at the normal rate during the experiment but that a further decrease in heart rate took place if the respiratory pump was switched off during the under water exposure In the present study this result was checked by

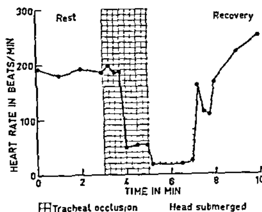


Fig 5 Summation of cardiac effects of asphyxia and water immersion

experiments in which asphyxiation preceded submersion An example is furnished in Fig 5 The tracheal cannula of a duck was occluded for 4 min and an asphyxia bradycardia of around 50 beats/min was established Then the bird was submerged The cardiac response was an additional slowing in frequency to less than 20 beats/min clearly showing a summation of the effects of asphyxia and submergence on the heart rate also in this case

Ventilation of lungs and air sacs during diving

I *Ventilation with air* In a series of seven experiments the asphyxia incurred during diving was relieved by ventilating the lungs and air sacs artificially with air The resulting effect on the heart rate is presented in Fig 6 The respiratory pump was set to perform 28 cycles/min which was the respiratory rate

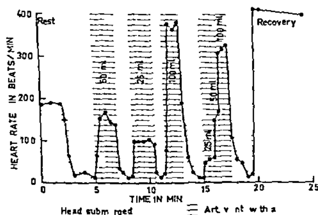


Fig 6 Artificial ventilation of lungs and air-sacs with air during diving

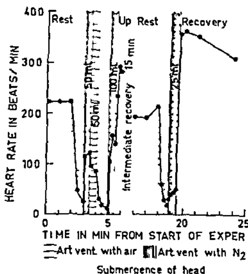


Fig 7 Artificial ventilation of lungs and air sacs with nitrogen during diving

of the duck at rest The volume of air delivered per cycle was varied as described below The pre diving heart rate was about 190 beats/min but after 1 min of submergence it had decreased to about 15 beats/min For the next 2 min of submersion this latter rate was maintained with only slight changes Exactly 3 min after the head had been immersed, artificial ventilation was started with a tidal volume of air of 50 ml Cardio acceleration followed immediately, and in *1 min the heart rate increased to 155 beats/min* whereupon *it decreased during* the next min to 130 beats/min at which rate it levelled off Upon discontinuation of the artificial ventilation the heart rate slowed down rapidly to the same low diving level as before Diving conditions prevailed for another 90 sec before the artificial ventilation was repeated The rate of the pump was the same but the tidal volume administered was only 25 ml Cardio acceleration to 100 beats/min resulted and as long as the artificial ventilation was maintained, the heart rate did not change appreciably When the pump was stopped however the normal diving bradycardia reappeared One min later artificial respiration was started for the third time now with a tidal volume of 100 ml, the rate being 28 cycles/min as before This degree of ventilation of the lungs and air sacs caused the duck to start breathing and brought about a marked tachycardia of 380 beats/min which closely resembled the cardiac response normally observed upon emergence The experiment was repeated after a new intermediate period of diving but with the difference that the three tidal volumes used were administered in succession starting with 25 ml and passing directly on to 50 ml and finally to 100 ml The number of cycles performed by the respiratory apparatus was still 28 cycles/min The previous relation between heart rate and tidal volume was confirmed and upon ventilation of the lungs

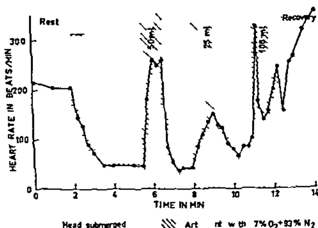


Fig 8 A ufical ventilation of lungs and air sacs with 7 ° O in nitrogen during di ing

and air sacs with 100 ml per stroke the apnoea was again abolished As a control the pump was stopped once more in order for the diving bradycardia to return, before surfacing was permitted Upon emergence a conspicuous tachycardia was elicited reaching 400 beats/min in only 15 sec. Whereas it was observed in this experiment and in several others that tidal volumes of air of around 100 ml usually caused a diving duck to resume breathing artificial ven tilation with about 50 ml of air per stroke of the pump would rarely induce breathing in submerged ducks and a tidal volume of 25 ml of air per breath was never found to produce such an effect

II Ventilation with nitrogen The experiments described in the previous section were also carried out using nitrogen in three cases instead of air Typical results are shown in Fig 7 From a pre-diving rate of 220 beats/min the cardiac frequency slowed down to 25 beats/min when the animal had endured water immersion for 2 min Ventilation of the lungs with 50 ml of nitrogen per "breath at a rate of 28 cycles/min caused a cardio-acceleration to 120 beats/min For one half min artificial ventilation with nitrogen was continued during which time the heart rate remained fairly steady at the same level In order not to deprive the bird completely of oxygen air was then given for the next 30 sec at the rate specified above for nitrogen This produced a retardation in the cardiac rhythm to 88 beats/min When the pump was stopped the heart rate decreased to 12 beats/min The respirator was next adjusted to give 100 ml of nitrogen per cycle and started again Cardio-acceleration to 160 beats/min followed rapidly with a subsequent fall to about 140 beats/min shortly afterwards At this point an intermediate recovery was allowed in order not to loose the animal A post-dive tachycardia of 280 beats/min was observed and after 15 min the resting heart rate was restored The head of the duck was then submerged

again, and a diving bradycardia of 20 beats/min was developed in 45 sec Artificial ventilation of the lungs and air sacs with 25 ml of nitrogen was administered, which brought about an increase in the heart rate to about 50 beats/min, the post diving tachycardia culminated at 300 beats/min

Submerged ducks were never seen to start breathing during artificial ventilation of the lungs and air sacs with nitrogen

III Ventilation with 7 % O_2 and 93 % N_2 After about 5 min of submergence the lungs of a duck contain, on the average, roughly 7 vol % CO_2 and 7 vol % O_2, the remaining 86 vol % being N_2 and other respiratory inert gases (Fig 1) In order to relieve the hypercapnia at this point while maintaining the hypoxia six experiments were carried out in which the lungs and the air sacs of the diving birds were artificially ventilated with a gas mixture containing 7 % O_2 and 93 % N_2 The number of cycles per min performed by the respiratory pump was adjusted to the resting respiratory rate of the duck, and the volumes of gas delivered per stroke were varied as described below The results obtained in one of the experiments are shown in Fig 8 The pre dive, resting heart rate varied between 216 and 204 beats/min Water immersion of the head caused cardiac slowing to 50 beats/min in 90 sec Exactly 3 5 min after the head had been submerged artificial ventilation was started with a tidal volume of 50 ml The duck started breathing and a conspicuous cardio-acceleration followed which reached 240 beats/min in 30 sec The heart rate remained at this level with only slight variations until the respiratory apparatus was switched off after 1 min By this stoppage of the pump the bird responded with the usual diving bradycardia Ninety sec later artificial ventilation was carried out with a tidal volume of 25 ml whereupon the heart rate increased to a peak value of 150 beats/min The acceleration phase was however, prolonged to 1 min and the cardiac frequency was not maintained at the high rate for any period of time but dropped subsequently to roughly 125 beats/min When the pump was stopped the heart rate fell only very slowly and the previous level of diving bradycardia was not reached As a matter of fact after a gradual slowing to 66 beats/min the heart rate started to increase again Upon artificial ventilation with 100 ml of gas per stroke of the pump the cardiac frequency abruptly increased from 100 beats/min to 325 beats/min This large increase in the heart rate was not only due to the ventilation *per se*, but also to the fact that the bird started breathing as described before It soon gave up the respiratory efforts however and the heart rate dropped rapidly down to 140 beats min only to increase again when the ventilation continued and the bird again tried to breathe Throughout this experiment and all others in which this particular gas mixture was used the duck appeared very uncomfortable although the effect on the heart rate was conspicuous In many cases the heart rate was brought to a faster frequency when ventilated with this gas than with air (see Fig 11) The symptoms exhibited by the bird were intermittent attempts to breathe violent struggling efforts the ECG showed sometimes marked a

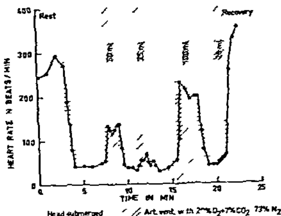


Fig 9 Artificial ventilation of lungs and air sacs with 20% O_2 and 7% CO_2 in nitrogen during diving

rhythmia and very frequently the heart rate did not slow down as usually upon discontinuation of the artificial ventilation (Fig 8 third diving period) Excessive ventilation of the lungs and air-sacs of the diving duck with this gas mixture therefore appeared to be dangerous.

II Ventilation with 20 % O_2, 7 % CO_2 and 73 % N_2 In this series of six experiments the hypoxia incurred in about 5 min of submergence was relieved, whereas the hypercapnia was maintained The results of an experiment are shown in Fig 9 The pre-diving heart rate fluctuated between 250 and 290 beats/min probably due to handling of the animal in this period Diving conditions resulted in a level of diving bradycardia of 40 beats/min When artificial ventilation was administered with a tidal volume of 50 ml after 4 5 min of submergence the heart rate immediately increased to around 130 beats/min The lungs and air sacs were ventilated artificially for 90 sec Upon switching off the pump the diving bradycardia reappeared and was even a little more pronounced than before After another 2 min of diving the respirator was again started delivering 25 ml of gas per stroke This time the cardio-acceleration was less marked the heart rate reaching a maximal value of 70 beats min after 1 min which was however not maintained throughout the entire period of artificial ventilation. The heart rate slowed down to the usual diving level when the artificial ventilation was discontinued Finally 3 min later the lungs and air sacs were ventilated with 100 ml of gas per stroke Initially this caused the heart rate to increase to 230 beats/min but a drop in the heart rate to 200 beats/min followed shortly afterwards The latter heart rate was maintained as long as the artificial ventilation continued Normal diving bradycardia resulted when the pump was stopped Before emergence was allowed venti[lation] with the tidal volume of 25 ml was repeated confirming the result first [mentio]ned Surfacing brought about the conspicuous tachycardia usually [observe]d upon ascent

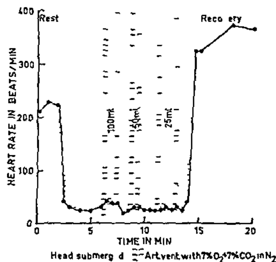


Fig 10 Artificial ventilation of lungs and air sacs with 7 % O_2 and 7 % CO_2 in nitrogen during diving

Artificial ventilation with this gas mixture gave relatively small effects in terms of cardio acceleration but the diving duck appeared less uncomfortable when it was used than with any other gas or gas mixture tested as judged by the quietness exhibited during diving and the general appearance afterwards

The submerged duck was never seen to resume spontaneous ventilation in to artificial ventilation of the lungs and air sacs with this gas

(V) *Ventilation with 7 % CO_2, 7 % O_2 and 86 % N_2* Artificial ventilation of the lungs and air sacs with air nitrogen or the gas mixtures described above does not only alter the composition of gas in the respiratory organs during a dive it also causes rhythmic stretching of these organs Because the activity of pulmonary stretch receptors are known to influence the heart rate (HEYMANS and NEIL 1958) it was decided to study the effect of the mechanical stretching of the respiratory organs *per se* by ventilating the lungs and air sacs after 4—5 min of submergence with a gas mixture containing 7 % CO_2 7 % O_2 and 86 % N_2 The results from one of these experiments are shown in Fig 10

The resting heart rate varied between 210 and 278 beats/min Water immersion of the head caused a bradycardia of 25—30 beats/min After 4 min of diving artifical ventilation was administered with a tidal volume of 100 ml The heart responded with an increase in frequency to roughly 40 beats min Upon discontinuation of the artificial ventilation the heart rate decreased to about 20 beats/min When the respirator was started again delivering 50 ml of gas per stroke the cardiac rhythm increased to 30 beats/min The respirator was stopped after 1 min and the heart rate slowed down to 24 beats/min Ninety sec later the lungs and air sacs were ventilated with a tidal volume of 25 ml This did not produce any significant cardio-acceleration at all The

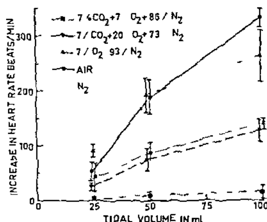


Fig 11 Comparison of the effects produced on the heart rate by the various gases or gas mixtures used for artificial ventilation during diving Means and ranges for each series of experiments are shown.

tachycardia upon emergence was very marked reaching a peak value of 470 beats/min Spontaneous respiration was never produced in a submerged duck during artificial ventilation with this gas mixture

11 Summary The results obtained in the experiments in which the respiratory organs of the birds were ventilated artificially during diving are summed up in Fig 11

It appears that ventilation with air and the gas mixture containing 7 % O_2 in nitrogen produced the largest effects in terms of cardio acceleration especially when tidal volumes of 50 and 100 ml were administered nitrogen was found to be the third effective gas In fact 25 ml of nitrogen per breath accelerated the cardiac rhythm nearly as much as air did but when larger tidal volumes were used, the effect of nitrogen was much less Somewhat less potent than nitrogen was the mixture consisting of 20 % O 7 % CO_2 and 73 % N_2 whereas ventilation of the lungs and air sacs with 7 % O 7 % CO and 86 % N was almost without any effect at all

Discussion

HUXLEY (1913) and LOMBROSO (1913) did not observe any pronounced asphyxic bradycardia in the duck upon clamping of the trachea In the present investigation however a conspicuous decrease in the heart rate resembling that seen during submergence was clearly demonstrated in response to such a procedure

Although asphyxia alone produces such a marked cardiac slowing one can be almost certain that the diving bradycardia is not initiated by the asphyxiation incurred For whereas the cardiac rhythm starts decreasing immediately upon water immersion of the head even if the resting pulmonary ventilation remains unchanged (ANDERSEN 1963) it usually takes about one half min of

Table I[1] *Changes in the asphyxic condition by artificial ventilation of respiratory organs during diving*

	Air	7% O_2 in N_2	N_2	20% O_2 + 7% CO_2 in N_2	7% O_2 + 7% CO_2 in N_2
Hypercapnia	−	−	−	0	0
Hypoxia	−	0	+	−	0

[1] Condition relieved — Condition unchanged 0 Condition made worse +

tracheal occlusion before the asphyxic bradycardia develops (Figs 2 3 and 5) This delay is probably the reason why LOMBROSO (1913) did not observe any cardiac slowing in his experiments for it appears that he subjected the animals to asphyxia for only a relatively short period of time HUXLEY s subjects exhibited a slight bradycardia but also in her studies the experimental period was shorter than those used in the present investigation Furthermore she worked to a large extent on decerebrates and it may well be that the animals have not been in very good condition Her method for recording heart rates was also much inferior to those available today

In a previous investigation (ANDERSEN 1963) it was shown that the physiological adjustment to diving are elicited by the actual submergence of the beak of the duck probably due to stimulation of certain receptors in this area This, however does not necessarily imply that the asphyxia is of no importance for the development of the cardio-vascular modifications during diving For it has been clearly demonstrated in this study (Fig 5) as well as in the previous one (ANDERSEN 1963 Fig 4 C) that the cardio retarding effects of water immersion *per se* and asphyxia summate algebraically regardless of whether asphyxia precedes water immersion or *vice versa* Therefore the very marked bradycardia seen during prolonged dives is apparently not due to the one factor or the other but to the combination of the two

As to whether the asphyxia bradycardia is determined by hypercapnia or hypoxia it seems clear from the results reported in the present paper summarized in Fig 11 and Table I that hypercapnia is by far the most important factor From Fig 11 it appears that the largest effects in terms of cardio-acceleration were obtained when the lungs and the air-sacs of diving ducks were artificially ventilated with air or a gas mixture composed of 7 % O_2 in nitrogen pure nitrogen being the third most effective gas Table I shows that ventilation with these three gases or gas mixtures have one common denominator the hypercapnia is relieved The CO_2 sensitivity of the mechanism is strikingly emphasized by the finding that ventilation with nitrogen had a more pronounced effect on accelerating the cardiac rhythm than had the gas mixture consisting of 20 % O_2, 7 % CO_2 and 73 % N_2 This indicates that even a quite severe degree of hypoxia

does not constitute an effective brake on the heart rate whereas accumulation of carbon dioxide certainly does

The mechanism is not entirely independent of the partial pressure of oxygen in the respiratory organs however for it has been shown that when the hypercapnia was relieved by artificial ventilation with air, 7 % O_2 in nitrogen or pure nitrogen the effect on the heart rate was largest when the hypoxic condition was improved (air) somewhat smaller when it was maintained unchanged (7 % O in nitrogen) and smallest by far when the hypoxia became increasingly severe (N_2) Furthermore the oxygen sensitivity was not manifest only upon marked oxygen deficiency because when the hypercapnia was kept constant and the supply of oxygen was varied the heart rate increased much more when 20 % O_2 and 7 % CO in nitrogen was given than when 7 % O_2 and 7 % CO in nitrogen was administered (Fig 11 Table I)

When the latter gas mixture is used for artificial ventilation of the lungs and air sacs after 4—5 min of under water exposure the composition of gas in the respiratory organs is supposedly not appreciably changed (Fig 1) Therefore the only factor introduced by this procedure is a rhythmic stretching of the lungs and air sacs Since this mechanical component of the artificial ventilation was found to exert a slight effect only in terms of cardio-acceleration (Figs 10 and 11) a few remarks may be made as to the abolition of the diving bradycardia

It was discussed in a previous paper (ANDERSEN 1963) whether the abrupt cardio-acceleration upon emersion results from stretching of the lungs and air sacs or from an increase in the venous return As long as the subject is breathing spontaneously these mechanisms may both act simultaneously However when artificial ventilation is administered the situation is different For whereas under spontaneous breathing the thorax cavity is enlarged due to the contraction of the respiratory muscles so that the intra thoracic pressure drops the pressure in the abdominal cavity increases and the venous return is consequently facilitated During artificial ventilation however this pressure difference between the thoracic and abdominal cavities is eliminated or reversed and blood tends to be squeezed away from the venous side of the heart Since the passive mechanical ventilation of the lung and air sacs during diving had such a small effect on the heart rate whereas ventilation with air (relief of asphyxia) or 7 % O_2 in nitrogen (relief of hypercapnia) made the submerged duck start spontaneous respiration and elicited a tachycardia similar to that seen upon emersion it appears likely that an increase in venous return which probably takes place upon emersion is more important for the abolition of the diving bradycardia than is the stretch of the lungs and air sacs An alternative explanation is however that the stretching of the respiratory organs have a very modest effect on the heart rate only when the respiratory center is inactive which may be the case in diving This latter argument is supported by the findings of ANREP *et al* (1936 a b) who reported that the heart

rate is influenced over the vagus center, which is inhibited directly by impulses arising in the lungs during their inflation as well as by impulses originating in the respiratory center And in addition the vagus centre is indirectly affected by the lungs through the HERING BREUER reflex

Finally it appears that spontaneou respiration is not resumed during diving unless the asphyxia is relieved or, at least that the hypercapnia is relieved *and* the hypoxia is not too severe This observation fits perfectly with that of ORR and WATSON (1913) who demonstrated that the respiration of ducks is inhibited by carbon dioxide excess so that apnoea may result from breathing gas mixtures containing more than 5 % CO_2

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Fluctuations in the Mitotic Frequency of the Glandular Stomach and Intestine of Rat under the Influence of ACTH, Glucocorticoids, Stress and Heparin

By

Tono Rasanen

Recei ed 9 November 1967

Abstract

Rasanen T *Fluctuations in the mitotic frequency of the glandular stomach and intestine of rat under the influence of ACTH glucocorticoids stress and heparin* Acta physiol. scand 1963 *58* 201—210 — Adrenocorticotrophin in a large single dose in contrast to the small doses produced a distinct drop in th mitoses of the gastric mucosa of rat but not in those of the duodenal mucosa The reducing effect of dexamethasone prednisolone and cortisol on the mitotic index was biggest for dexamethasone and smallest for cortisol The change in th mitot index was smaller in the duodenal epithelium than in the gastric mucosa Peritonitis produced a slight drop ir mit ses in the gastric and duodenal epithelium An acute stress produced a distinct reduction in mitoses in the gastric but not in the duodenal jejunal and colonic epithelium Heparin produced a distin t redu tion of mitoses in the gastric epithelium but not in the duodenal epithelium The reduction in the mitotic frequency is attributed to more rapid cellular changes in the gastric than intestinal lamina propria A factor reducing mitotic frequency in the gastric mucosa is probably the local rapid heparin liberation from the mucosal mast cells The reduction in mitotic frequency following various insults was great in the epithelium of the gastric mucosa slight r or nonexistent in the intestinal mucosa. This is attributed to more rapid changes in the lamina p the gastric mucosa than in the intestinal lamina propria. factor reducing mitotic frequer cy in the gastric mucosa is the local, true heparin liberated from the mucosal mast

The rat's glandular stomach has two zones of cell proliferation (STEVENS and LEBLOND 1953, BERTALANFFY 1960 1962) The cells splitting in the isthmus of the glands replace the cells removed from the surface epithelium The new mucous neck cells develop in the neck region of the glands It is hardly likely that any demonstrable renewal occurs in the chief cells and the parietal cells (TEIR SCHALMAN and SUNDELL 1952 LEBLOND and WALKER 1956)

The regeneration zone in the intestinal mucosa consists of the epithelium of the crypt from which the cells move towards the tip of the villi and finally detach themselves into the intestinal lumen (RAMOND 1904), although they lose some of their structure prior to detachment (CREAMER, SHORTER and BAMFORTH 1961)

The turnover time of the glandular stomach epithelium in rat is about 72 hours (STEVENS 1952) Measured by H³ thymidine the turnover time in the body of mouse stomach is over in one day (CREAMER et al 1961) In the glandular epithelium of the human stomach as evidenced by operative material from gastric ulcer and cancer cases the amount of mitoses exceeds that of healthy stomachs or duodenal ulcer cases (TEIR and RASANEN 1961) In the same pathological conditions no fluctuations in mitotic activity were noted in the mucosal epithelium of the duodenum (BERTALANFFY and NAGY 1961) the mitotic activity increased however with the degenerative mucosal changes of the jejunum in non tropical sprue (PADYKULA et al 1961)

The turnover time of the mucosal epithelium of the small intestine is 36 hours for the rat (LEBLOND and STEVENS 1948) the corresponding time for the mouse is 24—60 hours depending on the length of the intestinal villi (CREAMER et al 1961) The mitotic rate in the gastric mucosal epithelium of rat varies according to the nutritional condition of the organism though it is not directly affected by a meal (HUNT 1957)

ACTH seems to inhibit the experimental wound healing of gastric mucosa (MYHRE 1960) but seems on the other hand to have an increasing effect on the number of epithelial mitoses by rat (RASÄNEN and TEIR 1961) Glucocorticoids reduce the mitoses of the gastric mucosa (TEIR and ISOTALO 1953) The rate of reduction depends on the biological activity of the glucocorticoid in the pinnal epidermis of mice (CHAYDRY HALBERG and BITTNER 1956) The decrease in mitoses in the gastric epithelium is probably transient since prolonged use of glucocorticoids provokes hyperplasia of the gastric mucosa (BAKER and BRIDGMAN 1954) and intensified differentiation as the parietal cells increase (REID HACKETT and WELBOURN 1961) Prolonged stress produces hyperplasia of the gastric mucosa (COX and BARNES 1945 CAMBEL and SOLORIS 1952) although temporary stress reduces the number of mitoses

The changes in the exterior milieu of the epithelial cells of the mucosa and its effect on mitotic homeostasis are probably reflected in part in the cell changes in the lamina propria The present study was undertaken therefore to observe the changes in the granulation of the obviously fixed mast cells of

the lamina propria and in the mitotic activity of the parenchymal cells under the influence of stress ACTH glucocorticoids and heparin heparin is likely to be liberated from mast cells on their degranulation

Material and method

The test animals employed for the various experiments of the investigation were male rats of Wistar strain 4 months old all from the same animal farm. Before the experiment started the rats were allowed to adapt themselves to laboratory conditions for 10—14 days during which they were fed a mixed diet with water ad libitum The detailed arrangement of each test is described separately

Tissue specimens were fixed in Bouin s fluid and 4 per cent fresh lead acetate The 4 μ thick paraffin sections fixed in Bouin s fluid and hemalaun eosin stained were the preparations from which the mitoses were counted 10 μ thick sections fixed in the latter way and stained with toluidine blue were used for the mast cell counts Every effort was made to cut the section at right angles to the mucosa

The mitoses of the gastric body mucosa were counted at the height of the n ck of glandular tubes per 4 000 epithelial cells parallelly with the mucosa from contiguous fields of vision magnification × 945 and oil immersion in the way described in detail elsewhere (RASANEN and TEIR 1961) The mitoses of the mucosa of the small intestine were counted from the bottom of the crypts those of the large intestine at two-thirds of the height of the glandular tubes from 20 contiguous fields of vision parallelly with the surface of the mucosa with the above magnification

The mast cells of the gastric mucosa were counted in the way described previously (RASANEN 1960) The mucosal mast cells of the small intestine were counted from their most profuse zone from 20 contiguous fields of vision magnification × 945 and oil immersion

Wilcoxon s S-test was employed for the statistical treatment of the results

Results

1 Adrenocorticotrophin

ACTH in the form of a zinc emulsion (Cortrophin Z Organon) was given to 9 rats mean weight 172 g (range 176—224) 2 I U × 4 at 24 hour intervals intramuscularly The rats were decapitated 24 hours after the last injection The controls 10 rats of a mean weight of 179 g (range 168— 212) were given 0 5 ml saline injections during the same period The animals fasted for the last 12 hours

In the second experiment 10 rats were given a single dose of 15 I U of water soluble ACTH (Cortrophin Organon) the mean weight of the animals was 164 g (range 151—180) The 10 controls mean weight 169 g (range 146—188) were given one 0 5 ml saline injection The injections were intramuscular The rats fasted from the time of the injection only and were decapitated 5 hours afterwards

4 × 2 I U of ACTH at 24 hour intervals increased slightly the numbe the mitoses in the epithelial cells of the gastric and duodenal mucosa A single dose of ACTH produced a distinct decrease in the mitoses of the

Table I Medians of mitoses and of mucosal mast cells In the gastric mucosa mitoses are counted per 4 000 epithelial cells and mast cells per 1 mm² of tissue In the intestinal mucosa both are counted per 20 visual fields

	No of rats	Gastric mucosa		Duodenum		Jejunum		Colon
		Mitoses	Mast cells	Mitoses	Mast cells	Mitoses	Mast cells	Mitoses
ACTH 4 × 2 I U at 24-hr intervals	9	42	197 P < 0 001	85	–	–	–	–
Controls	10	35	1 062	133	–	–	–	–
ACTH 1 × 15 I U	10	9 P < 0 001	511 P < 0 05	92	234	107 < 0 05	215	35
Controls	10	26	1 042	78	291	77	252	21

mucosa (P < 0 001) but not in those of the duodenal mucosa, the latter could even increase slightly The increase of mitoses in the jejunum was almost significant (P < 0 05) The decrease in mucosal mast cells provoked by a small continuous dose of ACTH was significant in the gastric mucosa (P < 0 001), but slower per time unit than that following a large single dose of ACTH In 5 hours the mast cells of the gastric mucosa dropped by nearly a half (P < 0 05) The magnitude of the statistically demonstrable difference was not so clear because of the usually wide range of the number of mucosal mast cells No signs of degranulation of mast cells was noted in the duodenal and jejunal or colonic mucosa

2 Glucocorticoids

Dexamethasone (decadron phosphate Merck) was injected intramuscularly into 10 rats with a mean weight of 179 g (range 158—198) the dosage was 6 × 1 0 mg of the hormone with the acid component deducted at 8-hourly intervals Prednisolone (Di Adreson F Organon) and hydrocortisone (Solu Cortef Upjohn) was given in equal doses to 10 rats in two groups, mean weight 188 g (range 148—214) and 182 g (range 136—204) respectively The controls mean weight 179 g (range 168—212) were given 6 × 0 5 ml normal saline The rats were without food for 12 hours prior to decapitation which was performed 4 hours after the last injection The results are given in Table II

Under the influence of glucocorticoids the mitotic frequency of the glandular epithelium of the stomach dropped distinctly (P < 0 001) irrespective of the glucocorticoid used although the drop was greatest with dexamethasone The reduction in the mitoses of the duodenal epithelium was smaller and was biggest (60 per cent) in rats treated with dexamethasone (P < 0 001) With the other glucocorticoids the drop in mitoses of the duodenal epithelium was

Table II Medians of mitoses and of mast cells in the mucosa of the rat gastrointestinal canal

	No. of rats	Stomach		Duodenum Mitoses
		Mitoses	Mast cells	
Dexamethasone	10	1 P<0.001	51 <0.001	53 <0.001
Prednisolone	10	1.5 P<0.001	367 <0.001	75 <0.05
Cortisol	10	6.5 P<0.001	613 <0.01	95 <0.05
Controls	10	35	1.062	133

not so conclusive ($P < 0.05$). The degranulation of the mast cells of the gastric mucosa under the influence of glucocorticoids seemed to be significant ($P <$ 0.001—0.01).

3 Stress

A stress was created by peritonitis. 10 rats, mean weight 210 g (range 188—228) were each given an intraperitoneal injection of 1.0 mg of asbestos in sterilised aqueous suspension. Another group of 10 rats, mean weight 183 g (range 164—214) was given, in addition to asbestos, injections of 4×2 I.U. ACTH zinc once a day. The rats were decapitated 5 days after the asbestos injection and 24 hours after the last ACTH injection. The control rats were those employed in the former experiment.

An acute stress condition was created (1) by making 10 rats, mean weight 162 g (range 146—180) swim in 26° C water for 2.30—3.55 hours, decapitating them when they were on the point of drowning, or (2) by giving 10 rats, mean weight 167 g (range 150—178) intramuscular injections of 2 I.U. of insulin (Insulin Medica) and intraperitoneally 0.25 mg of parathion in ethanol solution. These and (3) the 10 rats, mean weight 168 g (range 148—195) injected with 0.125 mg of parathion, developed in 15—60 min a lethal shock and the rats were decapitated while they were in agony. Ten control rats, mean weight 169 g (range 146—188) were given 0.2 ml of normal saline intraperitoneally 1 hour before decapitation.

The results of these experiments are given in Table III.

A long term stress in the form of peritonitis provoked by asbestos seemed to produce a slight reduction in mitoses in the epithelium of the gastric mucosa ($P < 0.05$). In the duodenal epithelium, the reduction was slightly smaller ($P < 0.05$). Asbestos with ACTH reduced the mitotic activity of the gastric epithelium to some extent ($P < 0.05$), but not that of the duodenal epithelium. The mast cells of the gastric mucosa degranulated slightly ($P < 0.05$) as a result of peritonitis, profusely after ACTH ($P < 0.001$).

Table III Medians of mitoses and of mast cells in the mucosa of the rat gastrointestinal canal

	No of rats	Stomach		Duodenum		Jejunum		Colon Mitoses
		Mitoses	Mast cells	Mitoses	Mast cells	Mitoses	Mast cells	
Asbestos	10	17 P < 0.05	865 < 0.05	82 < 0.05	—	—	—	—
Asbestos + ACTH	10	10 P < 0.05	106 < 0.001	98	—	—	—	—
Controls	10	35	1 062	133	—	—	—	—
After swimming	10	4.5 P < 0.001	594 < 0.05	72	237	105 < 0.05	246	[illegible]8
Parathion + insulin	10	11 P < 0.001	390 < 0.001	91 < 0.05	307	88	273	31 < 0.05
Parathion	10	14 P < 0.05	874	67	273	65	316	33
Insulin.	10	16 P < 0.05	[illegible]91 < 0.01	77	278	106	239	2[illegible]
Controls	10	26	1 042	78	291	77	25[illegible]	[illegible]1

Table II Medians of mitoses and of mast cells in the mucosa of rat stomach and duodenum

	No of rats	Stomach		Duodenum Mitoses
		Mitoses	Mast cells	
After heparin	10	7 P < 0.01	1 114	9[illegible]
After heparin + ACTH	10	6 P < 0.001	278 < 0.001	[illegible]8 < 0.05
Controls	10	35	1 062	133

A rapidly developing stress with the rats swimming seemed to provoke a distinct reduction of mitoses in the gastric mucosal epithelium ($P < 0.001$) whereas this phenomenon was not seen in the epithelium of the intestinal mucosa In the jejunum the number of mitoses even seemed *to increase slightly during swimming* ($P < 0.05$) Nearly half the mast cells of the gastric mucosa degranulated during the swim ($P < 0.05$) No corresponding degranulation was noted in the mast cells of the duodenal mucosa

Parathion poisoning combined with insulin seemed to have a distinctly reducing effect on the mitoses in the gastric epithelium ($P < 0.001$) where the

mast cells degranulated rapidly ($P < 0.001$) In the duodenum and the colon the number of the mitoses of the epithelial cells increased to a weak degree ($P < 0.05$) The mast cells count of the duodenal mucosa seemed to increase slightly but *not significantly* With parathion alone using half the above dose the number of mitoses *in the epithelium* of the gastric mucosa decreased ($P < 0.05$) and so did the mast cells but not significantly Insulin shock alone also seemed to reduce the mitoses in the epithelium of the *gastric mucosa* ($P < 0.05$) and to induce mast cell degranulation ($P < 0.01$) No changes were demonstrable in the duodenal and colonic mucosa

4 Heparin

Heparin (Pularin Batch M 73990 Evans) was injected intra abdominally into 10 rats mean weight 197 g (range 178—216) 9×1.0 mg at 12 hourly intervals In addition 4×2 I U of ACTH zinc was injected into 10 rats mean weight 200 g (range 168—224) heparinized in the way described above the injections were made in the course of heparinization Three hours after the last heparin injection the rats were decapitated The controls were the same rats as in Table II The results of heparinization are given in table IV

Heparinization seemed to reduce distinctly the amount of *mitoses* in the epithelium of the gastric mucosa ($P < 0.01$) The administration of ACTH did not seem to change the inhibition of mitotic activity A slight reduction in the mitoses occurred in the duodenal mucosa with ACTH this became slightly significant ($P < 0.05$) Heparinization did not change the amount of mast cells in the gastric mucosa nor did it inhibit the degranulating effect of ACTH ($P < 0.001$)

Discussion

The mitotic index of the epithelial cells of rat gastric mucosa seems to be considerably more susceptible to changes than that of the duodenal jejunal or colonic epithelium The short term action of ACTH seems to reduce the gastric mucosal mitoses slightly It has been found to have a retarding effect on the regeneration of gastric mucosa (MYHRE 1960) Prolonged application of ACTH produces a marked increase in the amount of mitoses in the gastric mucosa of the rat (RASANEN and TEIR 1961) after the mucosal mast cells have almost completely lost their metachromatic granules (RASANEN 1961) Four-day treatment with ACTH provoked a slight increase of mitoses in the gastric mucosa in this study

The reducing effect of glucocorticoids on the mitoses may be attributable to the decreased activity of several enzymes of the cells (KOWALEWSKI 1962) and it results e g in reduced glucose consumption and energy production (BULLOUGH 1952) Yet the reduction in the mitotic index is greater in the epithelium of the gastric mucosa than in that of the intestinal mucosa which may possibly be due to a more rapid change during glucocorticoid action in

the lamina propria of the gastric mucosa than in that of the intestinal mucosa (RÄSANEN 1962)

A slight reduction in mitoses in the gastric and duodenal epithelium was noted during the stress due to peritonitis A local tissue lesion also reduced in its surroundings the mitotic activity of mouse epidermis (BULLOUGH and LAURENCE 1960) ACTH combined with peritonitis reduced the mitotic index of the epithelium of the gastric mucosa but not that of the duodenal epithelium The reduction in the mitotic index provoked by a short term, heavy stress was restricted to the epithelium of the gastric mucosa In the duodenal mucosa the number of mitoses even seemed to increase during short term stress At the same time a pronounced degranulation of mast cells was noted in the gastric but not the intestinal mucosa

The acetylcholine accumulating during parathion intoxication (SCHAUMAN 1960) may act as a histamine liberator (PATON 1957) and degranulate mast cells, which is probably associated with heparin liberation especially in the gastric mucosa Insulin is likely to enhance this effect with hypoglycaemia stimulating the vagus Although the insulin is bound in cellular nuclei (WILLIAMS ELGEE and LEE 1953) it does not seem to have a stimulating effect on the mitoses in the gastric mucosa apart from regulating the glucose metabolism. This effect may be inhibited by the heparin liberated from the mucosal mast cells, probably associated with histamine liberation and the stimulation of gastric secretion

Heparin seems to exert a reducing effect on mitoses in the epithelium of the mucosa but not in the duodenal epithelium Heparin inhibits the growth of tumour implants in mouse (JOLLES and GREENING 1960) and the mitoses in tissue cultures decrease under its action (CSABA KAPA MOLD and TORO 1961) Its inhibitory effect on the proliferation of cells is transitory (HEILBRUNN and WILSON 1949) and bound to the stereostructure of the heparin molecule (FISCHER 1936)

The inhibitory effect is probably due to the heparin inhibiting the action of cellular ribonuclease (ROTH 1953) Its effect was greatest on the gastric mucosa possibly because of the cellular structure in itself and heparin secretion through the gastric mucosa or because of the pH Heparin has a more pronounced inhibiting effect on the ribonuclease of the pancreas in an acid than in a neutral or alkaline milieu (ROTH 1953) The continuous local application of heparin in view of its effect as an inhibitor of mitoses may enhance the effect of cytostatics in tumour therapy (ESPINER VOWLES and WALKER 1962)

The disparity in the numerical variations of mitoses between the gastric and intestinal epithelium may be due to the fact that the mucosal polysaccharide which is possibly stored in the mucosal mast cells in gastric mucosa has a heparin structure (SMITH and GALLOP 1953) different e g from the duodenal heparinoid (BIANCHINI 1958) and perhaps to their different rates of liberation

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Acta physiol scand 1963 58 211—215

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The Effect of Various Dextran Fractions on the Suspension Stability of the Blood after Intravenous Injection in Cats

By

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Abstract

ELIASSON R and U SAMELIUS BROBERG *The effect of various dextran fractions on the suspension stability of the blood after intravenous injection in cats* Acta physiol scand 1963 *58* 211—215 — The effect of 3 different commercially available dextran preparations on the suspension stability of cat s blood has been studied after i v injection during circulatory experiments It is shown that dextrans with a mean molecular weight of 75 000 and higher induce such a marked decrease in the suspension stability that they can hardly be regarded as suitable plasma expanders for this species A dextran preparation with a mean molecular weight of 40 000 and a narrow molecular weight distribution (Rheomacrodex®) does not change the suspension stability of the cat s blood It has earlier been shown to be a suitable plasma expander and its use in physiological experiments on cats is therefore recommended

In many physiological experiments on animals it is common to fill the extra corporeal connections (tubes pumps etc) with an artificial colloid Dextran solutions are often used for this purpose

From the work of GRONWALL and INGELMAN (1945) THORSÉN and HINT (1950) and others it is known that dextran like other macromolecules can change the suspension stability of the blood THORSÉN and HINT (1950) showed that the increase in the sedimentation rate of the human red blood corpuscles (ESR) was proportional to the concentration and to the molecular weight of

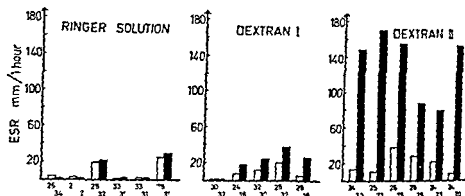


Fig. 1. The effect on erythrocyte sedimentation rate of various dextran preparations in normal saline and Ringer solution added in vivo to cat's blood. Each pair of piles represents the ESR before and after infusion of the solution in one cat. The figures below each pile give the corresponding hematocrit values.

the colloid. Below a certain molecular weight characteristic of each colloid there was, however, no increase in the ESR even at very high concentrations. This "critical molecular weight" was found to be about 60,000 for dextran. THORSEN and RICHTER (1959) showed that the structure of the dextran molecules was of no importance for the results.

A marked decrease in the suspension stability changes the rheological properties of the blood and may therefore introduce an experimental error in many circulatory studies. Since there are different dextran preparations available and apparently no investigation on the effect of these on the suspension stability of the cat's blood, it was thought of interest to study if there are any practical differences among these preparations when used in physiological experiments on animals.

Methods

Cats weighing 2.0—3.6 kg were anesthetized with Nembutal (30 mg/kg body weight, intraperitoneally). Blood was taken as soon as possible either by puncture of the heart or through a cannula in the femoral artery. All animals were then used for the ordinary investigations, i.e. circulatory studies. All tubes, which were connected with the blood vessels, were filled with the solution tested. The volume in the tubes was approximately 10 ml. In addition the animals received further 5—15 ml of the same solution during the experiment. At the end of the experiment (usually 3—6 hours) another blood sample was taken. The hematocrit was estimated by a micromethod and the tubes were centrifuged at 1 500 *g* for 20 min. ESR was determined according to the method of WESTERGREN (1926) using 4 parts of blood and 1 part of 0.1 M sodium citrate solution. The ESR values were measured after one hour. In those experiments where the hematocrit was changed this was performed by the addition of autologue plasma. The test solutions contained 6 per cent dextran (*Leuconostoc mesenteroides* strain B...) in normal saline or 5 per cent glucose. Three different preparations were used: dextran I — mean molecular weight ($\overline{M}_w$) 40 000 and 90 per cent of the molecules within 15 000—80 000; dextran II — $\overline{M}_w$ 75 000, 80 per cent within 25 000—1?0 000; dex-

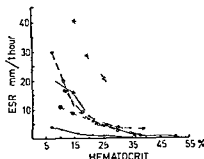


Fig 2 The correlation between erythrocyte sedimentation rate and hematocrit in blood from 6 cats

tran III — $\bar{M}_w$ 87 000 80 per cent within 25 000—170 000 The three preparations are commercially available as Rheomacrodex© Macrodex© and Vetridex© Pharmacia Ltd. Uppsala. Ringer solution was used as control

Results

No decrease in the suspension stability of the blood was observed when Ringer solution or dextran I were used The two other dextran preparations (dextran II and III) on the other hand caused a marked decrease in the suspension stability In many of the experiments the ESR was so high that maximum values were reached long before the 60 min had passed

The main results are given in Fig 1 The empty columns represent the ESR before infusion and the filled columns after the infusion of the various solutions. There was no difference between dextran in normal saline and in glucose Dextran III gave results almost similar to dextran II These data have therefore been omitted in the diagram

Readings of the ESR at periods shorter than 60 min have not been given In those cases where the ESR was very high the blood cells aggregated into large masses which settled at different speeds and therefore did not develop a distinct border line which could be used for the ESR value until the end of the 60 min period

The decreased suspension stability of the blood when dextran II or III were used also gave rise to disturbing sedimentation of the blood corpuscles in the transparent plastic tubes used as artificial anastomoses through the constant volume pump This was especially observed when the rate of blood flow was lowered for experimental reasons

It is known that the hematocrit values influence the ESR and therefore the relationship between these two parameters of the cat s blood was estimated In this work we only wanted to deal with the practical consequences of using different dextran solutions and therefore the relationship between ESR and the hematocrit in blood of different suspension stability was not studied From Fig 2 it appears that the changes in the hematocrit obtained in the actual experiments are not so great that they will significantly influence the interpretation of the results

Discussion

An increased erythrocyte sedimentation rate in vitro (ESR) is a function of a decreased *suspension stability of* the blood with *aggregation* of the red blood cells (FÅHRAEUS 1921) There is a close relationship between ESR and intravascular aggregation of the blood cells if attention is paid to the flow rate of the blood (PLOMAN 1920, FÅHRAEUS 1921, ODELL *et al* 1947 WEIS FOGH 1957 and others) There is no general agreement about the pathophysiological significance of intravascular aggregation Most of the controversy seems to be due to an ill defined use of the terminology *e g* the word sludge It appears likely, however that a marked intravascular aggregation increasing the relative viscosity of the blood, can cause a decrease in the capillary flow and intravascular settling of blood cells and probably also embolic occlusions of the small vessels, particularly when the flow is slow as in the postcapillary venules and in the sinusoids

It has been demonstrated that intravascular aggregation induced by different kinds of trauma hypothermia or by infusion of high molecular colloids (dextran, thrombin fibrinogen) is closely correlated with a decrease in oxygen tension of such vital organs as liver and kidney (HINSHAW *et al* 1960), degenerative changes in parenchymatous organs (FAJERS and GELIN 1959) pathological changes in ECG (SWANK and ESCOBAR 1957) a decrease in wound healing (ZEDERFELDT 1957) and a decrease in oxygen consumption (LÖFSTRÖM 1959) Most of these alterations can be prevented or reversed by proper treatment with colloids increasing the suspension stability of the blood

In those experiments in which dextran II or III were used a marked settling of the blood cells could macroscopically be observed in the transparent plastic tubes when the flow was slow The settling became especially disturbing on those occasions when for technical reasons the flow through a part of the extra corporeal system was shut off for a short period of time It is likely that the changes observed in the plastic tubes in part represent what occurs in the circulatory system (PLOMAN 1920 WEIS FOGH 1957)

Clinical dextran preparations of type II in this study have proven to be the plasma expander of choice in humans Due to a marked decrease in the suspension stability of the cat s blood produced by this dextran preparation it seems, moreover that it should not be used in experiments on cats where a normal circulation is of importance

Dextran preparations of type I do not induce a decreased suspension stability of the cat s blood and since it has been shown to possess good plasma expansion properties (GELIN *et al* 1961 DRAKE and LEWIS 1961) it appears suitable also for use in physiological experiments on cats

It has been shown that the effect of dextran on the suspension stability of blood from various species is highly different (ELIASSON and SAMELIUS BROBERG 1963) The results obtained in this investigation can therefore not be regarded as valid for other species than the cat

The dextran solutions were kindly suppl ed by Pharmacia Ltd Uppsala.

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Functional Organization of the Cuneocerebellar Tract in the Cat

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Abstract

HOLMQVIST, B., O. OSCARSSON and I. ROSÉN. *Functional organization of the cuneocerebellar tract in the cat.* Acta physiol. scand. 1963. 58. 216—235. — The experiments were performed on unanesthetized decerebrated and decerebellated cats with the cord transected at C3 except for the dorsal funiculi. Mass discharges were recorded from the dissected restiform body and unit recording was made from axons. It is shown that the cuneocerebellar tract contains two subdivisions: one consists of neurones monosynaptically activated by group I muscle afferents from one or a few muscles, the other of neurones disynaptically activated from cutaneous afferents. The latter subdivision contains three subgroups. a) Units activated both on movement of hairs and pressure on skin. These units receive disynaptic excitation from high threshold muscle afferents in addition to the excitation from cutaneous afferents. b) Units activated exclusively from pressure receptors in pads. c) Units activated exclusively from hair receptors. It is concluded that the cuneocerebellar tract and the dorsal spinocerebellar tract have a similar organization and are largely equivalent as channels for information from forelimbs and hindlimbs respectively. A notable difference is that the synaptic linkage to the exteroceptive subdivision is disynaptic in the cuneocerebellar tract and monosynaptic in the dorsal spinocerebellar tract. — The demonstration of a gracilocerebellar tract by GORDON and SEED (1961) has been supported.

It has repeatedly been suggested that the cuneocerebellar tract is a forelimb homologue to the dorsal spinocerebellar tract (DSCT) (BLUMENAU 1891, SHERRINGTON 1890, 1893, PASS 1933, FERRARO and BARRERA 1934, 1935 a, b, c, BRODAL 1941). This suggestion is supported by the recent observation of GRANT (1962 a) that it terminates in the forelimb areas of the cerebellar cortex in a manner resembling the termination of DSCT. Fibres of the latter tract reach exclusively the hindlimb areas (GRANT 1962 b). Both anatomical

(GRANT and REXED 1958 GRANT 1962 b) and physiological (HOLMQVIST, OSCARSSON and UDDENBERG 1963) investigations indicate that the DSCT has no forelimb component

The functional organization of the DSCT is known in considerable detail (LAPORTE, LUNDBERG and OSCARSSON 1956 a b LAPORTE and LUNDBERG 1956 HOLMQVIST LUNDBERG and OSCARSSON 1956 LUNDBERG and OSCARSSON 1956 1960 CURTIS ECCLES and LUNDBERG 1958 LUNDBERG and WINSBURY 1960 ECCLES OSCARSSON and WILLIS 1961) On the other hand, there is no previous investigation on the organization of the cuneocerebellar tract The present observations show that this tract and the DSCT have a similar organization though there are certain important differences

Methods

The experiments were performed on unanaesthetized precollicularly decerebrated and decerebellated cats The animals were paralysed with flaxedil and artificially ventilated The blood pressure was not allowed to drop below 70—80 mm Hg and was in most experiments between 90 and 120 mm The body temperature was kept between 36 5 and 39 C

The deep and superficial radial nerves were dissected bilaterally and mounted for stimulation in pools of warm mineral oil. The deep nerve was regularly cut but the superficial nerve often left in continuity with the periphery in order to permit natural stimulation of receptors In some experiments hindlimb nerves were also prepared for stimulation The stimuli were condensor discharges of short duration (half time of decay about 50 µsec) The stimulus strength is given as multiples of threshold for evoking a just perceptible ingoing volley or a just perceptible mass discharge in the rest form body The latter threshold value was used when the mass discharge appeared at a strength below that causing any visibl ingoing volley

The second cervical vertebra was delaminated and the spinal cord transected at the mid C3 level except for the dorsal funiculi which were left intact The tissue ventral of the fibrous septum between the dorsal and lateral funiculi was carefully split with the aid of two pairs of sharp forceps under inspection through a binocular dissection microscope The dissection resulted in a mm wide gap between the cord ends The cord was afterwards fixed in formalin and inspected for completeness of the transection

The ingoing volley was monitored by triphasic recording from the dorsal funiculi above the lesion In two experiments the fibres in the dorsal funiculi were stimulated half a cm below the lesion The cathode was placed on the dorsal funiculi and the anode in the muscles more caudally

Diagram] Fig 5 describes the recording conditions. A dissected restiform body was prepared as follows The cerebellum was sucked away leaving the peduncles and adjacent white matter intact The brachium conjunctivum and brachium pontis were cut through along the interrupted line A loop tied to the peduncles was hooked into one recording electrode and used for lifting the dissected restiform body from under lying tissue in order to produce a gap along the interrupted line (not shown) The other recording electrode was placed against the "dissected restiform body where it was in continuity with the brain stem at the rostral border of the eighth cranial nerve Mass discharges of 50 to 100 µV could be recorded on stimulation of the forelimb nerves. Fibres in the restiform body were recorded from with capillary microelectrodes as described by LAPORTE et al (1956 b) Two double beam oscilloscopes were used permitting simultaneous recording of several events

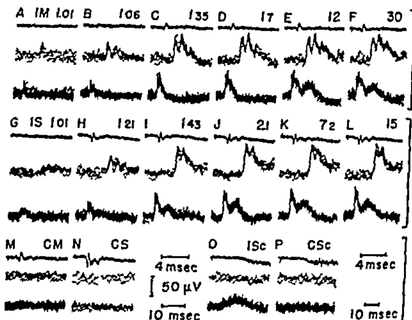


Fig 1 Mass discharges recorded from the dissected restiform body on stimulation of ipsilateral and contralateral muscle (deep radial) and skin (superficial radial) nerve in forelimb (IM CM IS CS) (A—N) and ipsilateral and contralateral sciatic nerve (ISc CSc) (O—P). The spinal cord was transected at C3 except for the dorsal funiculi Upper two traces in each set of records show on a fast time base the *ingoing volley recorded triphasically from the* dorsal funiculi at C3 and the mass discharge in the restiform body Lower trace shows the mass discharge on a slow time base Superposed sweeps. Stimulus strengths in multiples of threshold for evoking a mass discharge are indicated in A—L The stimulus strength in M—P was approximately 20 times threshold Dots mark stimulus artefacts Voltage scale refers to mass *discharge recording*

Results

1 *Mass discharge in restiform body*

Fig 1 shows discharges recorded from the dissected restiform body on stimulation of forelimb nerves after transection of the spinal cord except for the dorsal funiculi *Reasons for assuming that these discharges were evoked in the* cuneocerebellar tract will be given in the Discussion The upper trace in each set of records shows the ingoing volley recorded from the dorsal funiculi at the C3 level and the lower pair of traces shows the mass discharge in the restiform body recorded at different speeds A—F illustrate the discharge elicited on stimulation of the ipsilateral muscle nerve (IM) at indicated strengths The group II volley was not clearly visible in this experiment but appeared in other experiments at a strength slightly submaximal for group I (*cf* HOLMQVIST *et al* 1963) The first spike like discharge appeared always before any ingoing volley was discernible (A) The first two spikes were entirely due to excitation produced by the group I volley (A—D) the third spike was partly due to group I and partly to group II activation The relation between these three

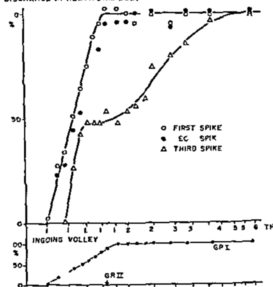


Fig 2 Correlation between stimulus strength ingoing volley and the three spike-like components of the mass discharge evoked by stimulation of ipsi lateral muscle nerve as in Fig. 1 The amplitude of the spike like components in per cent of maximal amplitude was plotted against stimulus strength (log scale on abscissa) in multiples of threshold for ingoing volley The left curve is drawn through the points representing the first spike The lower graph shows the amplitude of the in going group I volley in per cent of maximal amplitude plotted against stimulus strength (same abscissa as for upper diagram) The threshold for the group II afferents was determined at the end of the experiment by monophasic recording from the severed C7 dorsal root

spike like components of the discharge the stimulus strength and the ingoing volley is well documented by the curves shown in Fig 2 from a different experiment The first (open circles) and second (filled circles) spikes grew in parallel and became maximal at a strength submaximal for group II afferents (see lower graph) The third spike reached a plateau with the group I volley but increased further with additional activation of group II afferents

Records D and E Fig 1 show that activation of group II afferents also produced a large late activity which increased further with stimulation of group III afferents (*cf* E and F) Stimulation of the ipsilateral skin nerve (IS) evoked a large discharge in the restiform body (G—L) It appeared in this experiment before the ingoing volley was clearly visible In the other experiments a small volley was discernible before the mass discharge could be seen The contralateral nerves (CM CS) never evoked any appreciable mass discharge (M N)

The discharge elicited from low threshold muscle afferents had a latency of 1 0—1 3 msec in the various experiments when measured relative to the ingoing volley recorded at the C3 level This latency shows that the connection is monosynaptic (the conduction distance was about 2 5 cm) On the other hand the discharge evoked on stimulation of the skin nerve had a latency of 1 9 to 2 1 msec suggesting a disynaptic linkage

The synaptic linkage and the synaptic delay were further investigated by recording from the restiform body dissected at successively more caudal levels A—D Fig 3 show records obtained on stimulation of the muscle nerve at a



Fig 3 Recording of mass discharge from the restiform body dissected at successively more caudal levels (5 3 and 2 mm above obex and at obex as indicated) The spinal cord was transected except for the dorsal funiculi at C3 A—E obtained on stimulation of the ipsilateral muscle nerve (deep radial) at a strength slightly supramaximal for group I afferents. F—J were obtained on stimulation of the ipsilateral skin nerve (superficial radial) at a strength of about 3 times threshold E and J show the ingoing volleys recorded from the dorsal funiculi at C3 The distance between obex and recording site at C3 was 2 1 cm Superposed sweeps

strength slightly supramaximal for group I afferents In A the restiform body was dissected so as to be in continuity with the brain stem 5 mm rostrally of the obex There was no distinct presynaptic volley which is in agreement with the fact that the external cuneate nucleus does not extend more than 4 mm above the obex (GRANT 1962 a and personal communication) When the dissection was continued 2 and 3 mm further caudally (B C) the presynaptic volley became visible and it dominated the picture when the dissection was extended down to the obex (D) The presynaptic volley reached the C3 level 1 5 *msec (E) and the lower brain stem 1 8 msec after the stimulus The synaptic* delay can be calculated as 0 9 msec (*cf* A and D)

The presynaptic cutaneous volley grew in parallel with the muscle afferent volley (F—I) It reached the C3 level after 1 7 msec (J) and the lower brain stem after 2 0 msec (I) The discharge in the restiform body was evoked by the lowest threshold cutaneous afferents Hence the delay can be calculated as 1 8 msec (*cf* F and I) which indicates a disynaptic linkage The discharge in the intercalated neurones is presumably represented by the second spike like discharge that can be seen in G—I During the successively more caudal dissections of the restiform body the mass discharge evoked from the muscle nerve decreased relatively little (A—D) whereas the cutaneous discharge was markedly reduced in record H and possibly abolished in record I (the late potential in H and I is presumably due to activity in the main nucleus) These observations suggest that the anatomical path for the cutaneous impulses is different from that for the muscle impulses

4 g acilocerebellar tract GORDON and SEED (1961) made the unexpected observation that some cells in the rostral part of the gracile nucleus were antidromically invaded on stimulation of the ipsilateral cortex of the anterior cerebellar lobe These gracilocerebellar neurones were activated from very large cutaneous areas (mean 110 cm²) We have now obtained results which support these observations

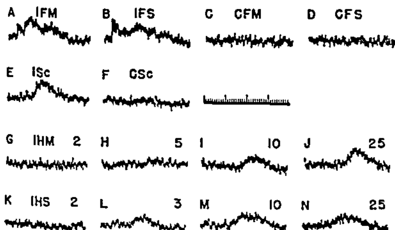


Fig 4 Mass discharges recorded from the dissected restiform body on stimulation of various forelimb and hindlimb nerves The spinal cord was transected except for the dorsal funiculi at C3 The following nerves were used ipsilateral and contralateral muscle (deep radial) and skin (superficial radial) nerve in forelimb (IFM CFM IFS CFS) ipsilateral sciatic (except hamstring) nerve (ISc) contralateral sciatic nerve (CSc) ipsilateral muscle (hamstring) and skin (sural) nerve in hindlimb (IHM IHS) The latter two nerves were stimulated at indicated strengths the other nerves were stimulated at approximately 20 times threshold The ingoing volleys (not shown) were recorded from the dorsal funiculi (forelimb nerves) and from the sciatic nerve proximally of the stimulating electrode Dots mark stimulus artefacts Time scale in msec

Records O and P Fig 1 were obtained on supramaximal stimulation of the ipsilateral and contralateral sciatic nerve (ISc and CSc) The ipsilateral but not the contralateral nerve produced a discharge in the restiform body The afferents responsible for this discharge were determined in the experiment illustrated in Fig 4 A—D show the typical discharges evoked on stimulation of forelimb nerves In G—J the ipsilateral hamstring nerve was stimulated at indicated strengths Stimulation of group I afferents did not evoke any discharge (G) but a small potential appeared with activation of group II muscle afferents (H I) and grew with additional stimulation of group III afferents (J) The discharge evoked on skin nerve stimulation appeared at a strength between 2 and 3 times threshold (K L) and grew at higher strengths of stimulation (M N) The relatively high thresholds may signify the need of considerable spatial summation Record E shows the discharge evoked on supramaximal stimulation of the ipsilateral sciatic nerve This discharge is not much larger than that evoked from the sural nerve This suggests convergence of many cutaneous afferents on to the same neurones, which may be correlated with the large receptive fields observed by GORDON and SEED (1961) The discharge evoked from high threshold muscle afferents (H—J) had a longer latency than the discharge evoked from cutaneous afferents which presumably is explained by differences in conduction velocity The short duration of the discharge evoked from the sciatic nerve may suggest occlusion between volleys in cutaneous and high threshold muscle afferents

2 Unit discharge in restiform body

Recording from fibres in the restiform body permitted an analysis of the unit activity contributing to the mass discharges described above All units

Table I Number of units encountered in the restiform body and classified according to their connections with afferents from indicated receptor types The classification is based on results obtained by electrical stimulation of the nerves (stretch receptor units being identified by their activation from group I muscle afferents) and/or adequate stimulation of receptors Number of units not tested with adequate stimulation are given within brackets See text

Identified by	Stretch receptors in muscle	Deep receptors in chest wall	Cutaneous receptors
Nerve stimulation	14 (+ 5)		(9)
Receptor stimulation	54	8	9
Nerve and receptor stim.	2		31
Totals	70 (+ 5)	8	40 (+ 9)

were recorded in the dorsolateral part of the restiform body just caudal to the eighth cranial nerve in the region indicated by a dotted line in Fig 5 J The fibres were encountered from the surface down to a depth of about 1 mm (the electrode was inserted perpendicularly to the floor of the fourth ventricle) and the various types to be described were intermingled at random In this part of the restiform body no cell bodies or presynaptic fibres were encountered

One hundred and thirty two units were recorded in 7 experiments and are classified into three groups according to their connections with different types of primary afferents (Table I) Electrical stimulation with graded strength of the deep and superficial radial nerves was tried with all units encountered Measurement of the central delay relative to the ingoing volley recorded at C3 often made it possible to determine if the linkage was mono- di or poly synaptic Stimulation of the dorsal funiculus at the C3 level permitted assessment of the synaptic linkage with a number of units not activated from any of the dissected nerves The deep radial nerve was always cut but the superficial nerve was in 6 experiments left in continuity with the periphery in order to permit activation from receptors The effects of receptor stimulation was tested with all the units encountered in these experiments except with 2 which were activated from the skin nerve but lost before adequate stimulation had been tried Stimulation of receptors was not tried in a preliminary experiment with all the nerves transected

Only 3 out of the 132 investigated units had no resting activity Those connected to undenervated receptive fields had usually a resting activity of 20 to 60 imp/sec The mean frequency was 33 and the range 0—120 The units with denervated fields i e most of the units activated from group I muscle afferents in the severed deep radial nerve had frequencies of between 0 and 60 impulses per second with a mean at 17 This suggests that the resting activity was partly due to a bombardment of impulses from the primary afferents The resting activity had often a relatively regular character but conspicuous bursts of spikes occurred in some units as is illustrated in Fig 7 F—J The "spontaneous

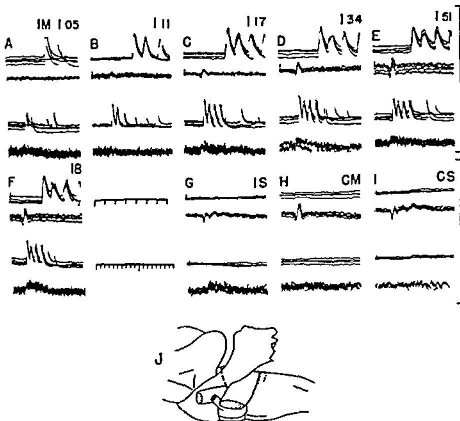


Fig 5 Cuneocerebellar unit activated from group I muscl afferents Upper two traces in each set of records show on a fast time base microelectrode recording from the axon and recording from surface of the dorsal funiculi at C3 lower two traces show on a slow time base microelectrode recording from the axon and recording from dissected restiform body A—F were obtained on stimulation of the ipsilateral muscle nerve (IM) at indicated strengths in multiples of mass discharge threshold. G—I show that no discharge was elicited by stimulation (at 20 times threshold) of the psilateral skin nerve (IS) or of the contralateral muscle and skin nerve (CM CS) Superposed sweeps Time scales in msec

The diagram (J) describes the recording conditions The dissected restiform body was prepared as follows The cerebellum was sucked away leaving the peduncles and adjacent white matter intact. The brachium conjunctivum and brachium pontis were cut through along the interrupted line A loop tied to the peduncles was hooked into one of the recording electrodes and used for lifting the dissected restiform body from underlying tissue (not shown) The other recording electrode was placed against the dissected restiform body where it was in continuity with the brain stem at the rostral border of the eighth nerve Axonal recording was performed within the area surrounded by the dotted line

burst in I consists of 23 spikes with a maximal frequency at 1 000 impulses per second Such bursts did not follow electrical stimulation of the dorsal funiculi (A—E) and there was no after-discharge following natural stimulation of receptors Possibly the bursts were due to excitatory action from other afferent systems than those ascending in the cord

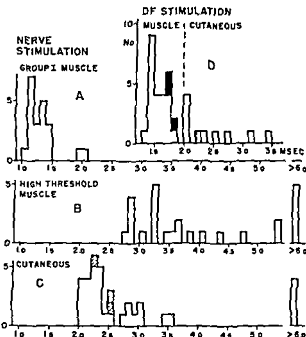


Fig 6 Latency of unit responses measured relative to the ingoing volley at upper C3 (histograms A—C) and latency of responses evoked on electrical stimulation of th dorsal fun culi at lower C3 (histogram D) In B the latency of the responses evoked by high threshold (group II and III) muscle afferents was measured r lative to the group I volley which at C3 precedes ie group II volley by approximately 0 9 msec (f HOLMQVIST *et al* 1963) In D the units to the left of the interrupted line were activated from muscle stretch receptors (black rectangles indicate three units activated from deep receptors in the chest wall presumably located in muscle) the units to the right were activated from cutaneou receptors Hatched rectangles in C ref r to units act vated only from pressure receptors in pads and stippled rectangles in C and D to units activated exclusively from ha r re eptors (see text)

1 *Units activated from stretch receptors in muscles*

These units were identified either by being activated from group I muscle afferents on stimulation of the severed nerve or by being activated on stretch of undenervated muscles but not on stimulation of cutaneous receptors Fig 5 shows a unit which discharged one or two spikes at a stimulus strength that evoked no perceptible ingoing volley (A) Four and sometimes five spikes were elicited by a submaximal group I volley (C) No additional spikes appeared when the strength was increased to supramaximal for group I (I I The ipsilateral skin nerve (G) and the contralateral nerves (H I) did not evoke any discharge

The remaining 20 units were similar The first spike appeared usually at a very low stimulus strength in 12 units out of 18 at a strength below 1 05 times threshold and only in one at a strength as high as 1 27 The repetitive response sho 5 1 maximal group I stimulation evoked 1 spike in

one unit 2 3 and 4 spikes in each four units 5 spikes in five units and 6 spikes in three units In no case was there evidence for additional activation from group II and III muscle afferents or from cutaneous afferents Afferent volleys in contralateral nerves were ineffective with these units as with all units en countered in the present experiments

The latency of the first spike relative to the ingoing volley at the C3 level was in most cases 1 0 to 1 4 msec proving a monosynaptic linkage (see histogram in Fig 6 A) In the unit shown in Fig 5 and one other unit the latency was longer, about 2 msec but the general similarity in behavior and virtual lack of need for spatial summation indicate that these 2 units were also monosynaptically activated It should also be mentioned that the unit in Fig 5 was found in an experiment with unusually long latencies the other 3 group I activated units had latencies of 1 4 msec

With 16 units activated from group I afferents in the deep radial nerve adequate stimulation of receptors was tested Only in two units was there an acceleration of the resting activity it was in both cases elicited by stretch of one of the muscles producing movement at the wrist Decrease of the resting activity was found in three units it was caused by movements at the wrist and pressure against deep tissues presumably muscle In one of these units strong pinching of the skin also caused inhibition but slight movement of underlying tissues could not be excluded

Fifty four units were identified by their excitation on stretch of and pressure against muscles These units were not activated from any of the dissected nerves but with the 20 units so tested electrical stimulation of the dorsal funiculi showed that the connection with primary afferents was monosynaptic (Fig 6 D Fig 7 K—M) In most cases a single muscle seemed to be responsible for the activation but it was often impossible to exclude that the effect was evoked from two or more adjacent muscles In 6 cases there was clear evidence for convergence one muscle of the upper arm and one of the forearm produced excitation There was never any excitatory effect on adequate stimulation of cutaneous receptors Inhibitory effects were not evoked from the skin on light stimuli but sometimes on strong pinching However unloading of muscles supplying excitation could not be excluded in these cases neither in the few cases where pressure or stretch of certain muscles caused some decrease of the resting activity

B Units activated from deep receptors in the chest wall

All of these 8 units except one had a resting activity which varied rhythmically with breathing They could be influenced by deformation of the chest wall but never by touch pressure or pinching of the skin In several cases pressure against subcutaneous tissue caused excitation from a receptive area of 40 to 100 cm^2 These units were not activated from any of the dissected nerves but

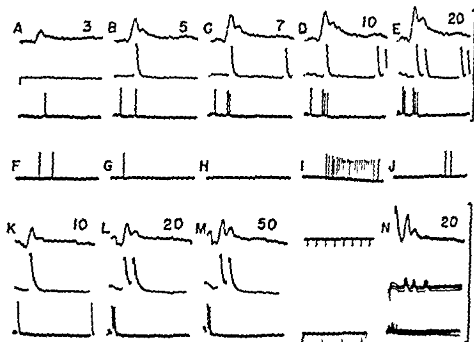


Fig 7 Three cuneocerebellar units activated by electrical stimulation of the dorsal funiculi at lower C3 A—J shows a unit activated from hair and pressure receptors in a large (about 100 cm²) receptive field on upper part of thorax F—J shows the "resting" activity during five successive sweeps K—M shows a unit activated from stretch receptors in the triceps muscle N (superposed sweeps) a unit activated from deep receptors in the chest wall Stimulation of the dorsal funiculi at indicated strengths (in multiples of threshold for evoking a mass discharge in the dissected restiform body The upper trace shows the mass discharge in the restiform body The two lower traces show the unit discharge at different speed Time scales in msec

were monosynaptically activated from fibres in the dorsal funiculi in the three cases so tested (Fig 6 D black rectangles Fig 7 N) Presumably these units were activated from group I muscle afferents in thoracic muscles

C Units activated from cutaneous receptors

These units discharged after a delay indicating a disynaptic or in a few cases a polysynaptic linkage when activated on electrical stimulation of the skin nerve (Fig 6 C) Nine of the units were identified by their responses on adequate stimulation of cutaneous receptors but were not discharged on stimulation of the cutaneous nerve (Table I) six of these units were tested by stimulation of the dorsal funiculi and shown to have a disynaptic linkage with primary afferents (Fig 6 D Fig 7 A—F) The 40 units tested with natural stimulation of cutaneous receptors have been divided into three subgroups according to their connections with various types of receptors (cf Table II) 34 units were activated from receptors responding to movement of hairs and

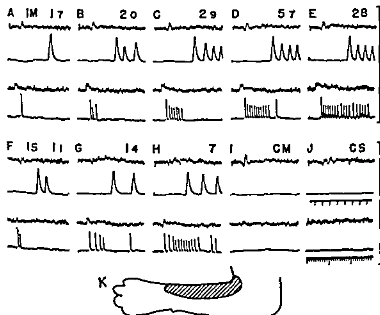


Fig 8 Cuneocerebellar unit activated from hair and pressure receptors The receptive field is shown in K A—E were obtained on stimulation of the ipsilateral muscle nerve (IM) and F—H on stimulation of the ipsilateral skin nerve (IS at indicated strengths I—J show that no effect was produced by stimulation of the contralateral muscle or skin nerve (CM CS) at about 20 times threshold The traces show ingoing volley at C3 mass discharge in restiform body and unit discharge at two speeds as in Fig 5 Time scales in msec

pressure on the skin 4 units were activated only from pressure receptors in pads and finally 2 units were activated exclusively from hair receptors

a Units activated both on movement of hairs and pressure on skin A typical unit is shown in Fig 8 A single spike appeared irregularly at 1 7 times threshold on stimulation of the ipsilateral muscle nerve (A) This strength was slightly suprathreshold for group I muscle afferents Further spikes appeared when the strength was increased to activate more group II afferents (B—D) and group III afferents (E) Stimulation of the ipsilateral skin nerve produced a similar train of spikes (F—H) The contralateral nerves were ineffective (I J) The diagram (Fig 7 K) shows the receptive area Within this area bending of hairs even blowing against them produced a marked increase in the resting activity Pressure against the skin increased the acceleration of the resting activity more than slight touch Pinching of a skin fold was not appreciably more effective than firm pressure

Many units activated from receptive fields (defined by natural stimulation) outside the area innervated by the dissected nerves were nevertheless discharged on electrical stimulation of these nerves However in these cases the excitatory action was weak and the spikes had sometimes a long latency For

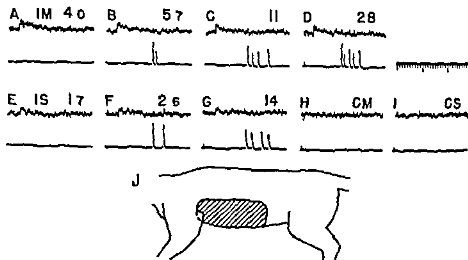


Fig 9 Cuneocerebellar neurone activated from hair and pressure receptors The receptive field is shown in J Upper trace shows the mass discharge in the dissected rest form body lower trace the unit response A—D were obtained on stimulation of the ipsilateral muscle nerve (IM) and E—G on stimulation of the ipsilateral skin nerve (IS) at indicated strengths H—I were obtained on stimulation of the contralateral muscle and skin nerve (CM CS) at a stimulus strength of about 20 times threshold Time scale in msec

example the unit shown in Fig 9 was activated on bending of hairs and pressure against the skin within the large area shown by the diagram (J) Stimulation of group II muscle afferents and cutaneous afferents evoked four or five spikes the first having a latency of 12 to 15 msec (relative to ingoing volley) This latency suggests that several interneurones were interpolated The weak excitatory effects coming from outside the receptive field may be of little functional significance Similar observations have been made with gracilis neurones (PERL WHITLOCK and GENTRY 1962)

Out of the 34 units investigated 26 were activated by a volley in the skin nerve and 22 by a volley in high threshold muscle afferents Possibly all units belonging to this group were activated not only from cutaneous afferents but also from high threshold muscle afferents and hence constitute one of the many pathways influenced by the flexor reflex afferents (ECCLES and LUNDBERG 1959 LUNDBERG 1959 HOLMQVIST LUNDBERG and OSCARSSON 1960, LUNDBERG and OSCARSSON 1961 1962) The initial spike discharged on electrical stimulation of the skin nerve had usually a latency of 2 0—2 5 msec (Fig 6 C) indicating a disynaptic linkage A disynaptic linkage may also be responsible for the initial spikes appearing at a slightly longer latency whereas a polysynaptic linkage is implicated in most cases when the first spike had a latency of more than 6 msec These spikes were usually discharged by a volley in relatively low threshold afferents (*cf* Fig 9) The initial spike produced by a volley in high threshold muscle afferents had a delay of 2 7 msec or more relative to the

Table II Classification of units activated from cutaneous receptors according to mode of activation and location of receptive fields Size of receptive fields

Subgroup activated	Location of receptive field	Number of units	Receptive field in cm²	
			Range	Mean
Both by movement of hairs and pressure on skin	Mainly foot	12	1.5—10	4.7
	Mainly forearm	1[illegible]	2—22	11.3
	Mainly upper arm	4	20—30	23.8
	Trunk	6	65—140	93.3
Only by pressure on pads	Pads	4	0.5—2.0	
Only by movement of hairs	Foot	2	0.7—1.0	

group I volley recorded at C3 (Fig 6 B) At that level of the cord the group II volley is delayed by about 0.9 msec relative to the group I volley (see *e g* Fig 2 in HOLMQVIST *et al* 1963) The initial spike was usually evoked by low threshold group II afferents and the latency indicates that they exert their excitatory action through one or more interneurones

A volley in cutaneous afferents evoked from one or a few spikes up to more than 30 The initial frequency was usually high when the latency of the first spike indicated a disynaptic linkage frequencies of 700—1 000 per second were observed A volley in high threshold muscle afferents usually evoked a slightly shorter train of spikes

All the units were activated by slight movement of hairs and pressure against the skin The effect evoked by movement of hairs had a fast adaptation, whereas the effect evoked by pressure had a slow and little marked adaptation From the description of cutaneous afferents by HUNT and MCINTYRE (1960 a b) it would appear that the units were activated both from fast adapting hair receptors and slowly adapting touch and pressure receptors Pinching sometimes increased the acceleration of the resting discharge produced by firm pressure of a skin fold However it is not clear if this indicates additional excitation from pain afferents or if it was due to an intense stimulation of pressure receptors Following stimulation of the skin (touch pressure or pinching) there was little or no after-discharge Light touch was usually effective from the whole area of the receptive field Only in one or two cases was there a narrow border zone in which only pressure and pinching were effective.

The size of the receptive field varied with respect to its position The average size increased from distal to more proximal regions of the limb and very large areas were found on the trunk (Table II) A similar organization has been found in other ascending pathways (*cf* MOUNTCASTLE 1957 MOUNTCASTLE and POWELL 1959 KRUGER SIMINOFF and WITKOVSKY 1961 LUNDBERG and OSCARS-

SON 1961, ANDERSSON 1962, PERL *et al* 1962) There was a tendency for the fields on the forelimb to be smaller than corresponding hindlimb fields in DSCT units (LUNDBERG and OSCARSSON 1960) which might be connected with a more finely graded coordination of movements in the forelimb A similar difference in size has been observed for forelimb and hindlimb receptive fields in cortical cells (ANDERSSON 1962 p 16)

The receptive areas on the trunk were large and extended in three cases from the level of the Th1 or Th2 vertebra down to the level of the Th10 (one unit) or L1 vertebra (two units) (*cf* Fig 9) The caudal extent is remarkable as the cuneate nuclei are said to receive afferents only from the dorsal roots cranially of Th6 to Th9 (the level variously given by FERRARO and BARRERA 1935 a WALKER and WEAVER 1942, LIU 1956) However, it should be remembered that each root innervates a dermatome that extends over an area corresponding to several vertebrae (HEKMATPANAH 1961)

The strong effects evoked from cutaneous receptors made it impossible to investigate any effects on adequate stimulation of group II and III muscle afferents However, it is of interest that pressure against deep tissues outside the area covered by the cutaneous receptive field did not produce any marked excitation or inhibition This might suggest that the deep receptive field is covered by the skin field

Inhibitory effects were only encountered in three units In these cases pinching of the skin outside the receptive field produced a slight decrease of the resting activity The inhibitory area was close to but did not surround the excitatory area

b Units activated from pressure receptors in pads Four units were activated exclusively from pressure receptors in pads Very slight pressure was sufficient to evoke strong excitation pinching did not produce any additional activation The effect showed little if any adaptation The receptive field was one or part of one pad and there was no excitatory effect from the surrounding hairy skin. Slight inhibition was evoked by pinching of the skin surrounding the pad in one unit no inhibition was observed in the other three units Two of the units were activated by low threshold afferents in the dissected skin nerve after a latency indicative of a disynaptic linkage (Fig 6 C hatched rectangles) In no unit was there any effect from the muscle nerve

c Units activated exclusively from hair receptors A distinct group seems to be formed by two units activated exclusively from hair receptors in small receptive fields One unit was activated from a 0 7 cm² large area at the distal end of the fourth toe the other from a slightly larger area just behind the central pad. The adequate stimulus was movement of hairs and the effect had a very fast adaptation The resting activity returned to its original value on pressure or pinching immediately after the initial burst of activity It is concluded that these units were activated exclusively from fast adapting hair receptors (*cf* HUNT and MCINTYRE 1960 b) No inhibitory effects were observed The first

unit discharged on electrical stimulation of the skin nerve and the second on stimulation of the dorsal funiculi (Fig 6 stippled rectangles) in both cases the latency suggested a disynaptic linkage Stimulation of the muscle nerve did not influence the units

Discussion

Anatomical considerations

It is generally conceded that fibres ascending in the dorsal funiculi terminate in the gracile, the main cuneate and the external cuneate nuclei but in no other brain stem structures (FERRARO and BARRERA 1935 a WALKER and WEAVER 1942 POMPEIANO and BRODAL 1957 *cf* however RANSON DAVENPORT and DOLES 1932 CORBIN and HINSEY 1935 concerning the termination of the rostralmost cervical roots) It is further assumed that the gracile and main cuneate nuclei send their axons up the brain stem through the crossed medial lemniscus whereas the external cuneate nucleus sends its axons ipsi laterally through the restiform body to the cerebellar cortex (FERRARO and BARRERA 1935 b BRODAL 1941 MATSKE 1951 JANSEN and BRODAL 1958) In our experiments the discharge in the restiform body was investigated after transection of the spinal cord leaving the dorsal funiculi intact According to the classical anatomical picture the recorded discharge would occur in the tract arising from the external cuneate nucleus However some caution should be exercised when excluding the main cuneate nucleus as a possible source for some of the cuneocerebellar fibres GORDON and SEED (1961) recently showed that some cells in the rostral part of the gracile nucleus send their axons ipsi laterally to cerebellum and the existence of a gracilocerebellar tract is strongly supported by results in our experiments Obviously the projection of the gracile and cuneate nuclei need to be reinvestigated with anatomical methods

Two groups of neurones were encountered in the cuneocerebellar tract One was monosynaptically activated from group I muscle afferents and the other disynaptically from cutaneous afferents Following cerebellectomy or transection of the restiform body all or almost all cells in the external cuneate nucleus degenerate indicating that they all project to cerebellum (FERRARO and BARRERA 1935 b) This might suggest that the intercalated neurones of the cutaneous pathway are located in the main cuneate nucleus However there is at present no anatomical evidence for any fibre connections between the main and external cuneate nuclei (JANSEN and BRODAL 1958)

Functional considerations

It is of special interest to compare the organization of the cuneocerebellar tract with that of the DSCT These two tracts have been considered as functional homologues forwarding information from the forelimbs and hindlimbs respectively (see Introduction) Our results show that the similarities in

ganization of the two tracts are striking but there are also *important differences* Each major functional group in the cuneocerebellar tract corresponds to a similar group in the DSCT

The *proprioceptive subdivision* of the cuneocerebellar tract consists of a relatively homogeneous group of neurones They are *monosynaptically activated from* group I muscle afferents but little, if at all, influenced from other types of afferents The response evoked by a volley in group I *afferents is repetitive* possibly because of a prolonged monosynaptic transmitter action as in the corresponding DSCT neurones (ECCLES *et al* 1961) *The convergence of group* I afferents to single cuneocerebellar units is limited to the afferents from one or a few muscles

The units in the proprioceptive subdivision of the DSCT have similar characteristics (LAPORTE *et al* 1956 b LAPORTE and LUNDBERG 1956 LUNDBERG and OSCARSSON 1956 1960, HOLMQVIST *et al* 1956 LUNDBERG and WINSBURY 1960 ECCLES *et al* 1961) They are monosynaptically activated *from group I* muscle afferents in one or a few muscle nerves and the response is often repetitive Some of the group I activated DSCT units receive additional excitation from group II muscle afferents Additional excitation from these afferents was not observed in the cuneocerebellar tract but might have been concealed by the strong excitation from the group I volley

The *exteroceptive subdivision* of the cuneocerebellar tract contains two or three functional subgroups of neurones

One subgroup consists of units activated by light movements of hairs and pressure against the skin suggesting excitation both from hair receptors and pressure receptors Most or all of these units receive additional excitation from high threshold muscle afferents This subgroup of cuneocerebellar units is similar to a subgroup in the DSCT (LUNDBERG and OSCARSSON 1960) The DSCT units are activated by pressure and pinching and also by light touch and many of them receive additional excitation from high threshold muscle afferents Some differences between the subgroups in the two tracts may only reflect differences in the excitability of the preparations The cuneocerebellar units had a higher excitability as indicated by their higher resting activity This may explain why the cuneocerebellar units were strongly activated by movement of hairs from the whole receptive field whereas in the DSCT units the effect of light touch was sometimes absent and when present usually elicitable only from the centre of the receptive field Other observations however, suggest real differences in the organization The DSCT units but not the cuneocerebellar units had often a marked after-discharge This suggests that the cuneocerebellar units are relatively little influenced from complex interneuronal chains and not activated by fibres which discharge some time after the end of the stimulus such as the C-fibres (*cf* DOUGLAS and RITCHIE 1962) In this connection it is of interest that the cuneocerebellar units in contrast to the DSCT units were little if at all activated by pinching

A second subgroup of cuneocerebellar units was activated exclusively from slowly adapting pressure receptors in pads and corresponds to a virtually identical group of "pad units in the DSCT (LUNDBERG and OSCARSSON 1960) Two neurones in the present material may suggest a third subgroup of cuneocerebellar units exclusively activated from fast adapting hair receptors in small receptive fields No corresponding subgroup in DSCT has been demonstrated

The units of the exteroceptive subdivision of the cuneocerebellar tract have a disynaptic linkage with the primary afferents The possibility was suggested above that the intercalated neurones might be located in the main cuneate nucleus The properties of the neurones in the gracile and main cuneate nuclei are known from a number of investigations (GORDON and PAINE 1960 GORDON and SEED 1961 KRUGER *et al* 1961 PERL *et al* 1962) but there are, at present, not enough data to permit a detailed comparison with the properties of the cuneocerebellar units However it should be noted that some of the neurones in the gracile and cuneate nuclei are activated from low threshold joint afferents whereas we have found no joint subdivision in the cuneocerebellar tract Similarly no joint subdivision has been demonstrated in the DSCT (LUNDBERG and OSCARSSON 1960)

The suggestion that the cuneocerebellar tract is a forelimb homologue to DSCT has been substantiated by our results The two tracts are largely equivalent as channels for proprioceptive and exteroceptive information It is interesting that the two tracts nevertheless are built according to partly different anatomical plans In the DSCT both the proprioceptive and exteroceptive subdivision have monosynaptic connections with the primary afferents in the cuneocerebellar tract only the proprioceptive subdivision

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Acta physiol scand 1963 58 236—242

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Dependence of Glomerular Filtration Rate on Proximal Tubular Reabsorption of Salt

By

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Abstract

LEYSSAC P P *Dependence of glomerular filtration rate on proximal tubular reabsorption of salt* Acta physiol scand 1963 *58* 236—242 — The purpose of the present investigation was to study the relationship between the rate of glomerular filtration and the rate of proximal tubular reabsorption of filtrate in mammalian kidneys In the exposed left kidney of white rats the time required from ligation of the left renal artery until the proximal tubules have completed reabsorption of the luminal filtrate and the tubules have thus become occluded was observed This time interval named occlusion time observed directly in a stereomicroscope was measured with a stop watch and could be determined within plus or minus 2 sec Inulin clearances were determined in periods immediately preceding the measurement of the occlusion time A linear correlation was demonstrated between glomerular filtration rate and occlusion time and it is concluded that the proximal reabsorption of sodium is a T max type of process independent of but limiting the glomerular filtration rate

Micropuncture studies on *Necturus* and mammalian kidneys have shown that the proximal reabsorption of filtrate is an active transport of sodium, water and anions following passively But it remains an unsolved problem as to how the sodium transporting capacity of the proximal tubules and the filtration rate of salt and water are mutually adjusted According to the concept of HOMER W SMITH (1951 p 326—328) the active reabsorption process creates a rate-limiting concentration gradient across the tubular wall which is a function of the filtered load On the other hand BOJESEN (1954) concluded from his own studies on "dilution diuresis and from the studies of others on "pressure diuresis and "salt diuresis that the proximal saline reabsorption is independ

ent of the load i e is a process of T max type which in some unknown way is kept proportional to the renal plasma flow Even though these experiments showed that the rate of reabsorption remained constant in spite of increased filtered load it might still be postulated that the reabsorption is a function of the filtered load as in one way or another the process of reabsorption might have been inhibited — by the low colloid osmotic pressure per se in the experiments on dilution diuresis or by the increased intrarenal pressure per se in experiments on pressure diuresis — to exactly the same extent as the reabsorption might be increased by an elevation of the load Thus we were still without final proof that the proximal reabsorption of sodium is a T max type of process as suggested by BOJESEN

Methods

In the present investigation white female or male rats (about 250 g body weight) were anaesthetized intraperitoneally with pentobarbital[1] The left kidney was exposed and illuminated as described for micropuncture studies by GOTTSCHALK and MYLLE (1956) The left ureter was cannulated with a polyethylene catheter to the renal pelvis and a ligature placed loosely around the left renal artery dissected free from the renal vein. This procedure allowed a stable kidney function as indicated by reproducible clearances of inulin during more than one hour 1—1 5 ml isotonic saline was slowly given intravenously before priming with 15 mg inulin and followed by continuous infusion of 1 per cent inulin solution (0 15 mg/min) Within 30 min from priming an almost steady plasma level of inulin was attained Plasma samples were drawn from the right carotid artery and ureteral urine was collected in 2 or 3 periods (generally 5—10 min each)

In one series of experiments the renal artery was ligated immediately after the last collecting period and the tubular lumina examined in the stereomicroscope (magnification × 100) By this technique it is visualized that ligation of the renal artery is followed by a gradual occlusion of the proximal tubules until no lumina are left and the tubules are seen as light homogenous solid coils The occlusion time from arterial ligation until the proximal lumina have disappeared was measured with a stop watch and could be estimated within an accuracy of about plus or minus 2 sec

In another series of experiments instead of ligating the renal artery after the last collecting period the left kidney was snap frozen at —65 C in a freezing mixture of acetone and solid carbon dioxide within 1—2 sec after cutting the renal pedicle of the living rat Tissues were then transferred to absolute ethanol at — 20 C and left at this temperature for 6 days (freeze substitution) The temperature was then slowly raised to room temperature and ethanol was changed once Tissues were embedded in paraffin via methyl benzoate and benzene and sections cut at 3 microns and stained with periodic acid Schiff (Mac Manus) in order to visualize the brush border of the proximal tubules The internal diameters of proximal tubules and the cell thicknesses were measured in 50 transverse cut circular superficial cortical proximal tubules in each kidney by means of a micrometer ocular

Serum and urine concentrations of inulin for estimation of glomerular filtration rate were determined by the method of BOJESEN (195?) modified for microanalysis.

[1] The doses of pentobarbital given to female rats were 10 mg per 100 g body weight (0 4 ml i p of a solution of 100 mg in 4 ml destilled water) The doses given i p to male rats were 25 mg (1 ml) for the first 200 g body weight plus 2.5 mg for each 10 g body weight above 200 g

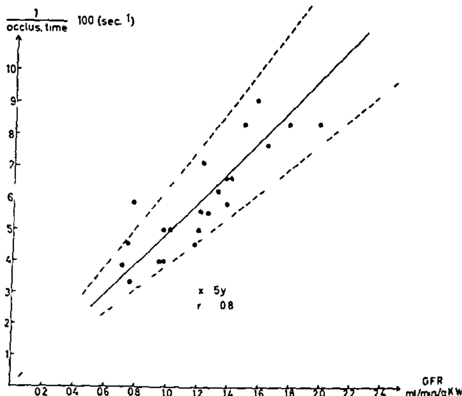


Fig 1 The figure shows the linear correlation between spontaneous variations in GFR and the reciprocal values for the occlusion time × 100 sec The broken lines are drawn corresponding to a maximal relative error of measurement of 12 5 per cent on both parameters

Results

As apparent from Fig 1 there is a close linear correlation between glomerular filtration rate and reciprocal occlusion time the correlation coefficient being + 0 8 The shorter the time required to occlude the tubular lumina the greater is the rate of glomerular filtration With infinite time of reabsorption, the regression line aims at a glomerular filtration rate of zero ml/min

Fig 2 shows the internal (luminal) diameters of the proximal tubules in kidneys with varying rates of glomerular filtration The mean tubular diameter in each kidney was determined with an error of less than 5 per cent (2 44—4 65 per cent) It appears from the figure that above filtration rates of 0 7 ml/min/g KW the internal proximal tubular diameter (and hence the proximal tubular fluid volume) does not differ significantly diameters *ranging from 26 to 28 μ* At filtration rates below 0 7 ml min/g KW the mean diameters decrease significantly with decreasing rates of filtration (t = 10 for the difference of mean diameters at 0 5 and 2 4 ml/min/g KW) However this is

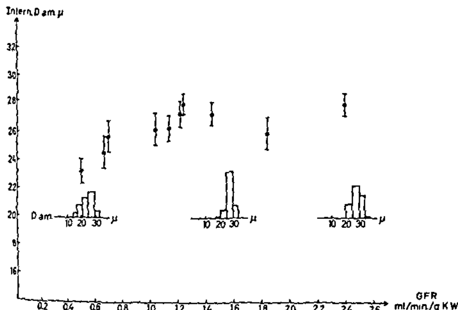


Fig 2 The figure shows the internal proximal tubular d ameter in relation to the glomerular filtration rate Each point represents the mean diameter of 50 proximal tubules measured ± 2 SE is set off at each mean

The histograms shown below represent three typ cal distributions of 50 diameters measured Note the skew to the left at the low rates of filtration The histograms are drawn from histological p eparations of k dneys having rates of filtrat on before preparation of 0 68 1 45 and 2 40 ml min/g KW

(SE = standard error of the mean)

predominantly due to a skew distribution leaving the major part of the diam eters within the same range as the mean diameter in the other groups Also the cell thickness of proximal tubules were uninfluenced by the rate of filtration above 0 7 ml/min/g KW However at rates of filtration below 0 7 ml/min/g KW the cell thickness of the tubules with the least internal diameters was increased

A normal rat kidney of 0 8—1 0 g consists of about 30 000—32 000 nephrons with a length of proximal tubules of 1 cm (SPERBER 1944) the internal (luminal) radius of proximal tubules being somewhat above 10 μ (GOTTSCHALK and MYLLE 1956) in the present experiments ranging from 13 to 14 μ From this the amount of filtrate in the functioning proximal tubules may be calculated

$$(\pi\ r^2 \quad L \times 32\,000)/10^8 = 0\ 17\text{—}0\ 20\ \text{ml}$$

where r represents the radius and L the tubular length If the glomerular filtration rate is 0 8—1 0 ml/min in this kidney (1 ml/min/g KW) and if 80 per cent of the glomerular filtrate were reabsorbed in the proximal tubules

it should take 13—15 sec to occlude the tubular lumina provided that the reabsorption continues at an unaltered rate until no more filtrate is left As seen from Fig 1 this is fairly close to the findings the occlusion time being 20 sec (16—24 sec) when the glomerular filtration rate is 1 ml/min/g KW

Discussion and conclusion

Four variable factors evidently determine the measured rate of disappearance of tubular fluid from the proximal lumina following ligation of the renal artery 1) the rate of reabsorption of sodium (and consequently of water) 2) the volume of tubular fluid per unit tubular length (i e the tubular diameter) 3) the concentration of sodium in the tubular fluid, and 4) the presence or absence of other osmoticly active compounds (e g mannitol or urea)

A fifth factor of significance might theoretically be a flow of liquid from the proximal tubular lumina either to more distal segments of the nephrons or as a retrograde flow back through the glomeruli This however was investigated by ODD HANSSEN (1960) using ferrocyanide as an indicator of early post mortem displacement of tubular fluid In no case a retrograde flow was observed and ferrocyanide present in the upper 4/5 of proximal tubules seemed not to pass the bend of the loop of Henle Nor were there any increment in the luminar diameters in more distal segments and the pelvis of animals with tied ureters did never dilate Since the peritubular capillary pressures (and hence the interstitial pressures) and the proximal intratubular pressures are identical (GOTTSCHALK and MYLLE 1956) no significant force capable of driving fluid to more distal segments could be expected a priori

If therefore the measured occlusion time is to give any information about the rate of reabsorption of filtrate the other three variables must be kept constant

In the present investigation all rats had the same diet and water to drink ad libitum The operation procedure was identical for all animals used and infusion of 1—1 5 ml isotonic saline before priming was given in order to secure a constant degree of hydration and equal sodium concentrations in the glomerular filtrate Neither mannitol nor urea infusions were given during the experiments and plasma concentrations of inulin did not differ by more than a few mg per 100 ml plasma from one animal to the other The concentrations of slowly or nonreabsorbable substances in plasma thus have been constant and of insignificant order of magnitude It therefore seems justified to postulate that the tubular fluid concentrations of sodium and other osmoticly active substances have actually been constant from one experiment to the other

As internal proximal tubular diameters did not vary significantly above rates of glomerular filtration of 0 7 ml min g KW it can be excluded that changes in the volume of proximal tubular fluid have influenced the measured occlusion time/filtration rate relationship Below filtration rates of 0 7 ml min g

KW mean diameters decrease This however, is due to the skew distribution leaving the major part of the population with diameters equal to the mean diameter at higher filtration rates Since it is the occlusion of this major part which will determine the observed occlusion time the decreased mean diameter at low filtration rates does not lead to any demonstrable deviation of the correlation between occlusion time and glomerular filtration rate in the low range of filtration rates from that applying to the higher range Thus the variations found in occlusion time must have been due to variations in the rate of proximal reabsorption only

Measurement of pressures in proximal tubules of rats under the same experimental conditions as in the present investigation (GOTTSCHALK and MYLLE 1956) have shown that the average intratubular pressure in a single rat did not differ from the average pressure of the whole material (193 proximal tubules from 56 rats) nor did the range of pressures in the whole material differ from the range of pressures in a single rat It seems reasonable to believe that these rats have shown the same spontaneous variations in filtration rates as those found in the present experiments Thus in spite of such variations in filtration rates both proximal intratubular pressure (GOTTSCHALK and MYLLE) and proximal tubular fluid volume (present investigation) are not significantly different from one rat to another This is in agreement with the present finding that the proximal tubular cell thickness is uninfluenced by the varying rates of filtration (and consequently of varying rates of salt reabsorption) a subject which will be discussed in detail in a future paper In this connection may be mentioned the finding of MAC ROBBIE and USSING (1961) in the frog skin that procedures inhibiting the sodium pump may occur without changes in the cell volume indicating simultaneous changes in ion permeability However at filtration rates below 0 7 ml/min/g KW diameters decrease with decreasing rates of filtration At such low filtration rates the cell thickness of tubules with the least diameter was increased apparently representing tubules at an intermediate state of occlusion In parallel the intratubular pressure in single open proximal tubules among areas of occluded tubules in kidneys after bleeding (low filtration rates) were very low 3 mm Hg or below (GOTTSCHALK and MYLLE 1956)

The present experiments thus indicate that the proximal reabsorption of filtrate continues at an unaltered rate after cessation of filtration brought about by ligature of the renal artery strongly indicating that the rate of reabsorption is independent of the "filtred load or supply of salt to the tubular cells i e is a T max type of process

Since the rate of reabsorption of filtrate (sodium) is independent of the filtration process and since a close correlation exists between occlusion time (under these experimental conditions reflecting the rate of reabsorption only) and the glomerular filtration rate it is concluded that the rate of glomerular filtration is limited by the rate of salt reabsorption Thus clearances of inulin

within this range of values are not a measure of glomerular function or filtration pressure, but a measure of the intrarenal sodium turnover rate (STR) and should rather be so named

How the sodium turnover rate (STR) is adjusted to the renal blood flow and to the intrarenal hydrostatic pressures still poses unsolved and important problems

Recent experiments using the method described in the present paper have suggested that angiotensin is responsible for this correlation between renal blood flow and sodium turnover rate which will be the subject for a future paper

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Acta physiol scand 1963 58 243—249

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Lactic Acid Assay with L(+)lactic Acid Dehydrogenase from Rabbit Muscle

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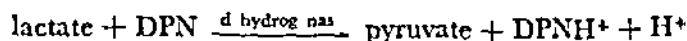
Abstract

LUNDHOLM L. E MOHME LUNDHOLM and N VAMOS *Lactic acid assay with L(+)lactic acid dehydrogenase from rabbit muscle* Acta physiol scand 1963 *58* 243—249 — Lactate assay with L(+)lactic acid dehydrogenase from rabbit muscle and with DPN involved the problem of quantitatively reducing lactate to pyruvate The problem was overcome by use of a solution with a high pH by an excess of DPN and by addition of a carbonyl reagent e g semicarbazide or hydrazine which trapped the formed pyruvate Hydrazine was found to be preferable to semicarbazide since it appeared to bind pyruvate more effectively It was thus possible to carry out the reaction with a 100 per cent recovery at pH = 9 0 This was advantageous because at pH > 9 0 hydrazine and semicarbazide rapidly formed products with DPN that absorbed at 340 mμ giving a high blank value that varied greatly with the pH of the solution and seriously affected the precision of the method Unlike hydrazine moreover semicarbazide reacted with formed DPNH in the presence of atmospheric oxygen, so that the extinction of DPNH decreased

For determination of lactic acid in large amounts of blood and tissues the method of FRIEDEMANN and GRAESER (1933) yields fairly reliable values but if only small quantities of lactic acid are available the method is not sufficiently sensitive For dependable results moreover considerable practical experience with the method is essential (LUNDHOLM MOHME LUNDHOLM and SVEDMYR 1963) The method of BARKER and SUMMERSON (1941) while more sensitive is marred by a substantial methodological error and does not consistently give quantitative recovery (LUNDHOLM et al 1963) The need of an accurate specific and simple method for lactic acid assay is therefore evident

Enzymatic determination of lactic acid was first described by LEHMANN (1938) who used lactic dehydrogenase from yeast with potassium ferricyanide as hydrogen acceptor. The prosthetic group in this enzyme is flavo cytochrome. The amount of potassium ferricyanide consumed can be determined either by redox titration (LEHMANN 1938) or by colorimetric means (WIELAND 1958). With the use of lactic dehydrogenase from yeast and potassium ferricyanide the lactic acid ⇌ pyruvate equilibrium is shifted entirely to the right. The drawback of this method is that yeast lactic dehydrogenase is difficult to prepare in pure form and is not yet commercially available.

Lactic dehydrogenase from skeletal muscle has DPN as cofactor and reduces lactate according to the reaction



$$\text{lactate} + \text{DPN} \xrightarrow{\text{l. dehydrogenase}} \text{pyruvate} + \text{DPNH}^{+} + \text{H}^{+}$$

At a physiologic or slightly alkaline pH the lactate ⇌ pyruvate equilibrium is shifted almost completely towards lactate (Fig. 1). PFLEIDERER and DOSE (1955) recommended for promoting the reaction towards the right a pH of 9.7 and an excess of DPN. The actual lactic acid content could then be calculated from the value for the equilibrium constant. HOHORST (1957) added a carbonyl reagent — semicarbazide — in order to bind the pyruvate produced and was thus able to convert lactate quantitatively into pyruvate. — Formed DPNH, however, reacts with semicarbazide in the presence of atmospheric oxygen and tends to give too low an extinction (Fig. 3). HOHORST (1957) therefore recommended that the reaction be carried out *in vacuo* at pH 10.5 for of quantitative recovery. SCHOLZ et al. (1959) suggested hydrazine for binding the pyruvate and HOHORST (1962) described a method using this principle. HOHORST also recommended that the enzymatic reaction should be performed at pH 9.5. — The requisite reagents are marketed under the name of Milchsaure Test Boehringer (Mannheim).

In the present investigation hydrazine evidently trapped the pyruvate more effectively so that the transformation of lactate into pyruvate occurred quantitatively at a lower pH than was the case with semicarbazide. This was advantageous in that semicarbazide and hydrazine in an alkaline medium (pH > 9.0) yielded products with DPN *which absorbed at 340 mμ. An alkaline medium* (pH ≷ 9.5) was thus associated with a high and variable blank value that appreciably influenced the precision of the method. Hydrazine moreover did *not react* with formed DPNH in the presence of atmospheric oxygen.

Methods

The extinction changes of the solutions at 340 mμ were determined by means of a Beckman DK 2 recording spectrophotometer. — A *dual beam instrument is greatly to be preferred in these experiments* since the extinction of the blank continuously increases. — The spectrophotometer was thermostatically controlled and the experiments were performed at 25° C. Matched 1 × 1 cm quartz cuvettes were used. *The* total fluid volume was 2.33 ml. *To one cuvette* was added on *determination of lactic acid*

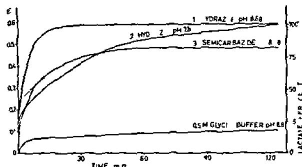


Fig 1 Determination of 20 μg L(+)lactate in 0 5 M glycine buffer without (undermost curve) and with 0 4 M hydrazine at pH 8 68 (curve 1) and pH 7 8 (curve 2) Curve 3 with 0 4 M semicarbazide, pH 8 68 Concentration of DPN = 2 3 10^{-3} M Reference cuvette without lactate

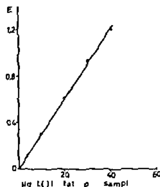


Fig 2 Relation between extinction at 340 mμ and the amount of L(+)lactate In the test cuvette 2 0 ml 0 5 M glycine buffer + 0 4 M hydrazine (pH 9 0) 0.2 ml 0 027 M DPN and 0 1 ml 3 perchloric acid with lactate In the reference cuvette the same solutions without lactate

2 ml 0.5 M glycine buffer containing 0 4 M hydrazine or 0 4 M semicarbazide — The pH of the solution was adjusted to 9 0 with NaOH unless otherwise stated 0 2 ml 0 027 M DPN (Boehringer) 0 03 ml L(+)lactic dehydrogenase (Boehringer) containing 2 mg enzyme protein per 1 ml and 0 1 ml 3 per cent perchloric acid containing varying amounts of Zn L(+) lactate H_2O To the reference cuvette were added all the solutions except zinc lactate On determination of lactic acid the extinction difference at 60 min and 25 C incubation was recorded Solutions were measured with calibrated Carlsberg pipettes The pH was determined with a glass electrode Both the DPN solution and the LDH solution somewhat lowered the pH of the buffer solution and a further pH fall occurred on reduction of lactate to pyruvate when free hydrogen ions were formed. The pH of the buffer solution was thus shifted downward The pH values recorded in the following text are, therefore the final ones the initial pH of the buffer solution having been 8 0 9 0 or 10 0

The molar extinction of DPNH was assumed to be 6 22 10^6 cm² per mole (KORNBERG 1957) An extinction increase of 0 1 would have been equivalent to 0 0161 μ mole DPNH or lactate per 1 ml cuvette contents Since the cuvette contained 2 33 ml solution an increase of 0 1 in the extinction was equivalent to 3 38 μg lactate per cuvette

Results

Lactic Acid Recovered at Different pH with Semicarbazide and Hydrazine

At pH 8 8 (9 0) only about 15 per cent of the lactate was converted to pyruvate in the absence of a carbonyl reagent (Fig 1) Addition of 0 4 M

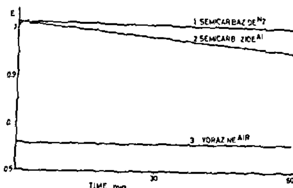


Fig 3 Reaction between semicarbazide and DPNH Reference cuvette without DPNH Wave length 340 mμ
Curve 1 1 9 10^{-4} M DPNH + 0 4 M semicarbazide in 0 5 M glycine buffer pH 9 5 The solutions saturated with N_2
Curve 2 The same as 1 but in air
Curve 3 1 10^{-4} M DPNH + 0 4 hydrazine + 0 5 M glycine buffer pH 9 0 in air



Fig 4 1) Immediate absorption at 340 mμ of 2 5 10^{-3} M DPN in 1 M glycine buffer at different pH
2) The same with 1 M glycine buffer + 0 4 M hydrazine
3) With 1 M glycine buffer + 0 4 M hydrazine + 0 03 ml LDH
The extinction was measured within 1 min after adding DPH to the solution and its pH was then determined

carbazide increased the recovery to 85 per cent whereas the recovery after 0 4 M hydrazine was 100 per cent (Fig 1) When the buffer solution pH was raised to 10 0 or 10 5 the recovery in the presence of semicarbazide or hydrazine usually exceeded 100 per cent but at these high pH levels the values varied appreciably i e the recovery was not uniform When the pH was lowered to 7 8 recovery was still 100 per cent after hydrazine but the reaction was conspicuously retarded to ensure a quantitative reaction within 60 min it was necessary to double the amount of LDH (0 06 ml) (Fig 1) Fig 2 illustrates experiments in which various amounts of Zn 1 (+) lactate were determined with 0 4 M hydrazine plus 0 5 M glycine buffer at pH 9 0 and after 60 min incubation The average recovery was 103 3 per cent and the standard deviation ± 2 per cent

Variations in DPN Content

In order as far as possible to lower the extinction of the reference cuvette various concentrations of DPN were tested at lactate determination On addition of half the usual amount of DPN (0 1 ml 0 027 M) the recovery was

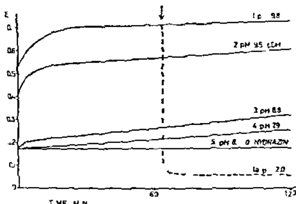


Fig 5 Reaction between $2.5 \cdot 10^{-3}$ M DPN and 0.4 M hydrazine + 0.5 M glycine buffer at different pH Reference cuvette without DPN Wavelength 340 mμ
Curve 1 pH 9.8 1a at the arrow 0.4 ml 5 N HCl was added to both cuvettes and pH was lowered to 2.0
Curve 2 The same as 1 but with addition of 0.06 ml LDH which decreased pH to 9.5
Curve 3 pH 8.8 Curve 4 pH 7.9
Curve 5 0.5 M glycine buffer (without hydrazine)

100 per cent With addition of only one fourth the usual amount however the recovery was 75 per cent Reduction of the amount of DPN was thus of limited value

Reaction between DPNH and Semicarbazide

At alkaline pH DPNH reacted with semicarbazide in the presence of atmospheric oxygen so that the extinction decreased (Fig 3) When the buffer solution was saturated with N_2 before addition of DPNH no decrease occurred Hydrazine even in the presence of atmospheric oxygen did not affect the extinction of DPNH

Extinction of DPN at Different pH and Effects of Hydrazine and Semicarbazide

It was sought to determine the cause of the wide range of values obtained on lactic acid assay at pH > 9.0 in the presence of hydrazine or semicarbazide DPN extinction at 340 mμ was found to be pH dependent and tended to increase greatly at pH ≥ 10 In the presence of hydrazine or semicarbazide the extinction increased substantially even at pH > 9.0 LDH had no effect on the extinction provided the coincident shift in pH was taken into account (Fig 4) In addition to this immediate increase of the extinction hydrazine and semicarbazide produced a continuous increase the rate of which mounted with rising pH (Fig 5) Extinction of the blank at 60 min was highly pH dependent On addition of *e g* 0.06 ml LDH to the buffer DPN solution the pH of the mixture fell from 9.76 to 9.48 – a circumstance which greatly influenced the extinction of the solution (Fig 5) It was not possible even by very exact measurement of the various components to ensure a constant and identical pH for on reduction of lactate to pyruvate free hydrogen ions were formed in amounts which depended on the quantity of lactic acid determined Thus the pH depression was 0.17 pH units greater for determination of 20 μ than for determination of 6.6 μg lactate The original pH of the buffer in these

experiments was 9 01 The extinction of the reference solution was pH dependent to a lesser degree in the pH 8—9 range than at higher pH values — a circumstance which probably accounts for the far greater consistency of the recovery at pH < 9 0 At pH < 9 0, however, 100 per cent recovery was recorded with hydrazine but not with semicarbazide — hydrazine thus being decidedly superior in this respect

The continuous increase of the extinction at 340 mμ which occurred in the presence of hydrazine and semicarbazide probably reflected the formation of a condensation product with DPN DPN has a marked tendency to form such compounds (KAPLAN 1960) When the pH of the solution was lowered from 10 to 2 by addition of HCl the extinction promptly returned to its original value at pH 2 (Fig 5), indicating that this hypothetical condensation product was rapidly hydrolyzed in acid media

Discussion

Hydrazine to all appearances trapped the formed pyruvate more effectively than did semicarbazide This circumstance was probably responsible for the conversion of all lactate into pyruvate even at neutral pH in the presence of hydrazine Since the propensity of both hydrazine and semicarbazide at 340 mμ to form absorbing compounds with DPN was greatly accelerated at pH > 9 0 it was unsuitable to perform the experiments at pH exceeding 9 0 where extinction of the blank became high and greatly dependent on pH In this respect our method deviated from that of HOHORST (1962) who recommended pH 9 5 Lastly semicarbazide reacted with DPNH in the presence of atmospheric oxygen so that the extinction tended to fall Hydrazine did not show this behaviour and was thus preferable to semicarbazide

With the use of hydrazine it was possible to determine *l (+) lactate* quantitatively indeed the recovery was somewhat over 100 per cent The reason for this is not clear Possibly the assumed molar extinction of DPNH was somewhat too low A slight systematic error may arise furthermore when different lactate concentrations are determined It is apparent from Fig 3 that the extinction of DPN increased somewhat between pH 8 0 and 9 0 Reduction of lactate was associated with the formation of free hydrogen ions which tended to lower the solution pH according to the amount of lactate reduced This tended in turn to decrease the extinction of DPN in the test cuvette relative to that in the reference cuvette thus lessening the extinction difference between the two This source of error could probably be reduced by increasing the glycine buffer strength to 1 M

In a further investigation the enzymatic method of determining lactic acid has been compared in respect to precision and quantitative recovery, with the methods of FRIEDEMANN and GRAESER (1933) and BARKER and SUMMERSON

(1941) in experiments on pure lactic acid solutions blood and extracts from smooth and striped muscle (LUNDHOLM et al 1963) This comparis.. demonstrated the superiority of the enzymatic method in sensitivity ..ccuracy specificity and quantitative recovery

This investigation was aided by grants from the Swedish M dical Research Council and the Research Foundation of the Swedish D abetes Association

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Acta physiol scand 1963 58 250—254

From the institute of Occupational Health Helsinki Finland

Turnover of Rat Serum Lipoprotein Cholesterol

By

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Abstract

Karvinen E and M Miettinen *Turnover of rat serum lipoprotein cholesterol* Acta physiol scand 1963 *58* 250—254 — The distribution of cholesterol-4-C^{14} in α_1 α_2 and β lipoprotein and chylomicron fractions separated by paper electrophoresis was studied from 3 hours to 8 days after oral administration of cholesterol-4 C^{14} The apparent turnover time of cholesterol-4 C^{14} in the lipoprotein and chylomicron fractions was found to be very similar in all fractions or about 79 hours

The turnover time of rat plasma cholesterol has been determined by Hotta and Chaicoff (1955) They injected cholesterol 4 C^{14} intravenously and found the turnover time of total plasma cholesterol to be about 60 hours On the other hand it is known that plasma cholesterol is divided in different fractions About 60 per cent of rat serum cholesterol is carried by α_1 lipoprotein 25 per cent by α_2 lipoprotein and 15 per cent by β lipoprotein when fractionated by paper electrophoresis (Miettinen 1957a) However the turnover of cholesterol contained in these various fractions of rat serum has not been studied The purpose of the present investigation was to determine the turnover of the cholesterol bound to the different lipoproteins of the rat serum

Material and methods

Male rats of the Wistar strain weighing 200 to 300 g were caged individually and given a stock diet for 3 weeks ad libitum Then the rats were fasted overnight and fed at 11 AM 8 μc of cholesterol-4 C^{14} in 2 g of fat free basal diet The labeled cholesterol was supplied by the Radiochemical Centre Amersham England The diet was prepared by dissolving cholesterol-4 C^{14} in ether and pouring on the dry basal diet Then the dry diet was made to a paste by adding water The rats ate the paste in about 15 minutes

From 8 rats blood samples of about 1 ml were taken under ether anesthesia from the tail 3 6 9 13 33 and 57 hours after the ingestion of the labeled cholesterol It has been

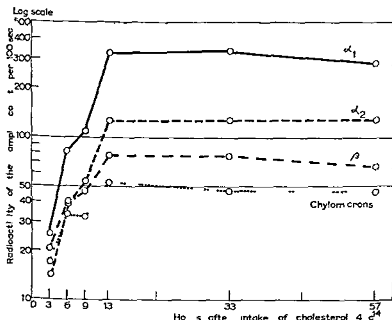


Fig 1 Semilogarithmic plot of the radioactivities of serum lipoprotein and chylom cron fractions after intake of cholesterol-4 C' (Experiment I)

reported (MIETTINEN 1957b) that bleeding produces an increase of cholesterol in α_2 and β lipoproteins In order to study the changes in the lipoproteins during the first 24 hours following the intake of cholesterol-4-C^{14} it was necessary to draw a relatively large volume of blood during a short period of time In order to find out how large was th effect of taking the volume of blood necessitated by the experiment another series of experiments was performed in which only one blood sample of about 1 ml was taken daily for 9 days after the ingestion of cholesterol-4 C^{14}

Fractionation of the serum lipoproteins was carried out according to the paper electrophoretic method of NIKKILA (1953) as modified and described elsewhere (MIETTINEN 1957a) using 0 5 ml of serum per analysis

The electrophoresis paper was cut into stripes containing albumin plus α_1 globulin plus α_1 lipoprotein α_2 globulin plus α_2 lipoprotein β globulin plus β lipoprotein and the area around the starting line containing chylomicrons The stripes were boiled in 10 % ethanolic KOH for 2 hours The unsaponifiable lipid was taken up in petroleum ether and washed with 50 ethanol and water The unsaponifiable lipid was transferred to steel planchets and assayed for radioactivity using a thin mica window Geiger tube (0th Century EW 3 H) and an EKCO Automatic Scaler N 530 F

Results

In Fig 1 and 2 the total radioactivity found in the unsaponifiable lipid fraction of α_1 α_2 and β lipoprotein and the chylomicron fraction of rat serum is shown as a function of time The apparent turnover time of cholesterol-4-C^{14}

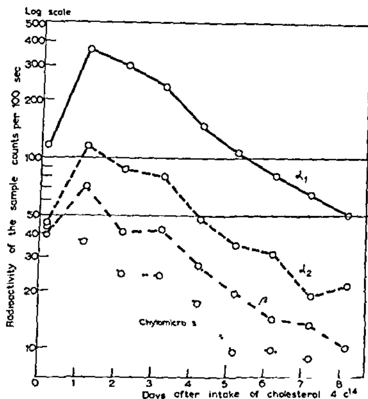


Fig 2 Semilogarithmic plot of the radioactivities of serum lipoprotein and chylomicron fractions after intake of cholesterol-4 C^{14} (Experiment II)

Table I Estimated relative specific activity of cholesterol bound in the different lipoproteins

Hours after cholesterol 4-C[1]	α_1	α_2	β	Chylo micron
5	1.9	1.9	2.8	44
29	5.8	4.8	5.0	37
53	4.8	3.7	2.9	24
77	3.8	3.4	3.0	24
101	2.3	2.0	[illegible]	17
1[illegible]5	1.8	1.5	1.4	9

[1] Total radioactivity (cpm) of the lipoprotein bound unsaponifiable material divided by the amount of cholesterol (mg %) in each fraction determined colorimetrically (MIETTINEN 1957a)

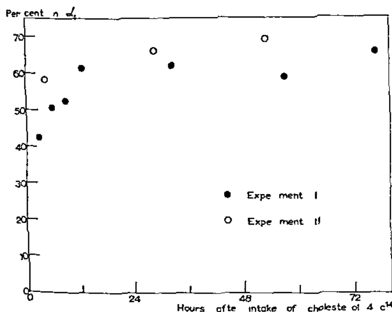


Fig 3 Radioactivity of α_1 lipoprotein lipid as per cent of the total activity in the lipoprotein lipid.

in the different lipoprotein fractions is estimated to be about 79 hours in group 2 (Fig 2) Excessive bleeding may have decreased the apparent turnover time Correction for the volume of the blood samples taken would give an apparent turnover time of about 89 hours

Table I shows the relative specific activities of the lipoprotein cholesterol fractions

Discussion

The percent distribution of radioactivity in the different lipoprotein fractions found in the present study is similar to the distribution of the amounts of total cholesterol in rat α_1 α_2 and β lipoproteins (MIETTINEN 1957a) It has been reported that bleeding produces a shift of cholesterol from α_1 lipoprotein to α_2 and β fractions in the rat (MIETTINEN 1957b) In order to avoid this shift only small blood samples were taken in the present Experiment II The results of Experiment II show no change in the distribution of cholesterol radioactivity during the experiment except an initial shift from the chylomicron fraction to the lipoproteins (Fig 2) The results of Experiment I do not show a much lower proportion of labeled cholesterol in the α_1 lipoprotein fraction than was the case in Experiment II (Fig 3)

The rather similar relative specific activities found in cholesterol bound to α_1 α_2 and β lipoproteins (Table 1) may indicate that there is a rapid equilibra

tion between the different lipoprotein fractions This finding is in agreement with the observations by FREDRICKSON and HAVEL (1958) that the exchange of labeled cholesterol between chylomicrons and lipoproteins occurs rapidly in the dog both in vivo and in vitro

The activity curves for the different lipoprotein fractions in Experiment II were straight lines when plotted on semilogarithmic paper This is in agreement with the findings of HOTTA and CHAICOFF (1955) who were able to plot similar straight lines for the total plasma cholesterol after an intravenous injection of a solution of cholesterol-4 C^{14} into rats An apparent turnover time of approximately 60 hours was found by these authors and by KARVINEN and MIETTINEN (1962) for the plasma cholesterol after the intravenous administration These estimates do not differ much from the present estimates for the apparent turnover times of cholesterol-4 C^{14} in the different lipoproteins and chylomicrons.

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Acta physiol scand. 1963 58 255—262

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Mast Cell Content and Fatty Acid Metabolism in the Epididymal Fat Pad of Obese Mice

By

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Received 21 November 1962

Abstract

Hellman B S Larsson and S Westman *Mast cell content and fatty acid metabolism in the epididymal fat pad of obese mice* Acta physiol scand 1963 *58* 255—262 — The changes in the mast cell content of the epididymal and subcutaneous adipose tissues were studied in two different types of obesity in mice Both with the obesity induced by goldthioglucose and that associated with the American variety of the obese hyperglycemic syndrome there was a considerable accumulation of mast cells in the fat depots While the relative number of mast cells was calculated as 3 per 100 epididymal fat cells in the lean controls it was more than [illegible]0 for the obese hyperglycemic animals This finding stresses the importance of expressing metabolic data on lipogenesis directly in terms of the fat cells and not per tissue weight nitrogen or DNA when comparing the adipose tissue in normal and obese individuals The greatly increased mast cell content of the adipose tissue in obesity would be consistent with previous work on the rat in which it was suggested that the lipase activity of the adipose tissue is concerned with accumulation of fat in depots However in so far as heparin did not influence the release of free fatty acids or clearing factor lipase from the isolated epididymal adipose tissue no *in vitro* effect of heparin was demonstrated

Previous studies have revealed some pertinent morphological and biochemical data about the epididymal adipose tissue in mice with the American variety of the obese hyperglycemic syndrome (Hellman Taljedal and Westman 1962 *b* Hellman Larsson and Westman 1962 a b Westman Larsson and Hellman 1962) In the static phase of obesity there was a marked redu

Table I The number of fat and mast cells in thousands per mm³ in the epididymal and subcutaneous adipose tissue from AN AO and ANO mice In addition the number of mast cells have been expressed per thousand fat cells Mean values ± S F Each group consisted of 9 animals

Animal	Epididymal adipose tissue			Subcutaneous adipose tissue		
	Number of fat cells in thousands per mm³	Number of mast cells in thousands per mm³	Number of mast cells per thousand fat cells	Number of fat cells in thousands per mm³	Number of mast cells in thousands per mm³	Number of mast cells per thousand fat cells
AN	6 7 ± 1 5	0 21 ± 0 06	29 ± 4	19 2 ± 4 7	0 57 ± 0 09	28 ± 4
AO	3 0 ± 0 6	1 57 ± 0 54	530 ± 170	2 8 ± 0 5	0 43 ± 0 06	187 ± 39
ANO	2 5 ± 0 3	0 75 ± 0 34	270 ± 100	4 6 ± 1 0	0 47 ± 0 09	117 ± 24

in the number of fat cells per unit tissue weight but no concomitant decrease in the relative nitrogen content of the epididymal fat pad as compared with lean litter mates (HELLMAN *et al* 1962 a) The importance of expressing the metabolic data in relation to the number of fat cells was particularly evident when analysing the *in vitro* conversion of acetate into neutral fat and fatty acids

In the present investigation the previously noted absence of a correlation between the number of fat cells and the nitrogen content was studied further by estimating the mast cell content of the epididymal fat pad of normal and obese mice The mast cells were chosen as representative for non fat cells since preliminary experiments revealed that the obese hyperglycemic syndrome was associated with a high proportion of mast cells within the epididymal adipose tissue Attempts were also made to evaluate the physiological significance of heparin by studies of its effect on the release of both clearing factor lipase and free fatty acids from the isolated epididymal fat pad of normal and obese hyperglycemic mice

Material and Methods

Adult male mice of the obese hyperglycemic strain from *R B Jackson Memorial Laboratories Bar Harbor Maine U S A* were used The mice were classified into the following 3 groups *1* Lean animals (Ob Ob or Ob ob) referred to as AN mice *2* Animals with obesity due to the hereditary obese-hyperglycemic syndrome (ob ob) and referred to as AO mice *3 Animals (Ob Ob or Ob ob) with obesity induced by a single intraperitoneal injection* of 1 7 mg/g body weight of goldthioglucose (S'z [illegible] *oleosum*) and referred to as ANO mice

Morphological analyses The left epididymal fat pad was removed with minimal [illegible] from 9 animals in each of the three groups and weighed on a torsion balance [illegible] tion a sample of subcutaneous fat from the gluteal region was taken The [illegible] procedure was performed throughout in a uniform manner and the number [illegible] calculated per mm³ on the basis of measurements in 200 μ thick [illegible] stained with hematoxylin-eosin (see HELLMAN *et al* 1962 a d) The [illegible] cells in the adipose tissue was estimated from cell counts within a

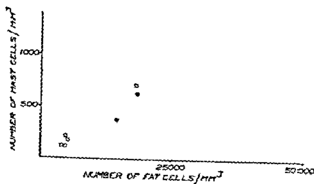


Fig 1 The relation between the number of fat and mast cells per mm³ in the AN mice □ = epididymal adipose tissue ● = subcutaneous adipose tissue

made on 10 different sections 10 μ thick and stained with 0 1 % toluidine blue (magnification 675 ×) The reduction formula given by FLODERUS (1944) was used for computing the actual values per mm³ from that observed in the sections (cf HELLMAN 1959) The error in the mast cell determinations amounted to about ± 20 %

In vitro release of lipase and free fatty acids In this part of the investigation 21 AN and 21 AO-mice were used The most distal parts of the epididymal fat pads (ca 100 mg) were placed in Warburg vessels containing 1 ml modified Krebs Ringer phosphate solution at pH 7 4 (see HELLMAN LARSSON and WESTMAN 1961) This solution was supplemented with 0 1 % glucose and 5 human serum albumin which contained approximately 1 6 mM free fatty acids (FFA) per mM albumin In half of the vessels heparin was added in a concentration of either 30 or 1 000 μg per ml The adipose tissue was incubated with continuous shaking at 37 C in an atmosphere of pure oxygen for one hour In determining the lipolytic activity an aliquot of each incubation medium was assayed for its ability to produce free fatty acids from a cotton seed oil substrate The procedure was similar to that used by CHERKES and GORDON (1959) except that cotton seed oil (Infonutrol) was used instead of coconut oil and the medium to be assayed for the lipase activity was mixed with the substrate in the proportion 1 3 to obtain a final pH of about 8 5 (cf KORN 1959) The free fatty acids thus evolved and the free fatty acid content of a part of the medium were determined with the modification of DOLE s (1956) method described by TROUT ESTES and FRIEDBERG (1960)

Results

The number of fat and mast cells per mm³ epididymal and subcutaneous adipose tissue is given in Table I In the AN mice the number of mast cells was calculated as 3 per 100 fat cells for each of the two types of adipose tissue Within this group of mice a positive correlation apparently existed between the number of mast and fat cells see Fig 1 The reduced fat cell number per unit volume of tissue in the obese mice was associated with a considerable increase in the relative number of mast cells as compared with the AN mice In the epididymal fat pad the number of mast cells per 100 fat cells was more than 50 for the AO mice and 27 for the ANO mice The corresponding value for the subcutaneous adipose tissue were 19 and 12 The appre

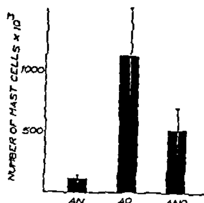


Fig 2 The mast cell content of the epididymal adipose tissue in the AN and AO mice Each bar represents the number of mast cells obtained per total epididymal adipose tissue when the specific gravity was assumed to be unity

Table II The in vitro metabolism of fatty acids in the epididymal adipose tissue from AN and AO mice The release (+) or uptake (—) of free fatty acids (FFA) as well as the lipase activity released from the adipose tissue in the presence or absence of heparin is expressed as μeq FFA per 100 mg wet weight and hour Mean values ± S E The number of animals studied is given within brackets

Animal	Release (+) or uptake (–) of FFA in the medium		Assay for lipase activity in the medium	
	30 μg heparin	no heparin	30 μg heparin	no heparin
AN (9)	+ 0 03 ± 0 07	+ 0 10 ± 0 08	0 11 ± 0 05	– 0 03 ± 0 21
AO (9)	– 0 05 ± 0 09	– 0 14 ± 0 19	0 13 ± 0 08	0 15 ± 0 19
	1 000 μg heparin	no heparin	1 000 μg heparin	no heparin
AN (12)	– 0 12 ± 0 08	+ 0 09 ± 0 09	0 03 ± 0 17	– 0 18 ± 0 11
AO (12)	– 0 11 ± 0 07	– 0 05 ± 0 07	0 15 ± 0 17	0 02 ± 0 15

of the mast cells within the epididymal fat tissue was also apparent when the figures were calculated as absolute values for both fat pads see Fig 2

The results obtained when the epididymal adipose tissues of the AN and AO mice were incubated either without heparin or with it in two different concentrations are given in Table 2 expressed as μeq FFA per 100 mg wet weight and hour In neither the AN nor the AO mice had the presence of heparin any effect on the release of FFA from the tissue The assay for the clearing factor lipase activity was negative even for the incubation medium which contained heparin

Discussion

During the last 15 years the concept of the metabolism of adipose tissue has been modified It is now generally agreed that adipose tissue represents an extremely active metabolic system The epididymal fat pad is of particular

interest in experimental diabetes on account of the very sensitive in vitro assays for insulin for which this tissue may be utilized (RENOLD et al. 1960). In a comparison of the lipogenetic activity of this type of adipose tissue from normal and obese hyperglycemic mice HELLMAN et al. (1962 a) discussed the importance of calculating the metabolic data as activity per fat cell and not only per unit tissue weight or nitrogen. The choice of parameter for the metabolic data is, however, irrelevant in the present experiment where the effect of heparin has been tested using tissue from the same animal as control. However, our observation of a greatly increased mast cell content of the adipose tissue in the AO mice seems to clarify the somewhat puzzling finding that the relative nitrogen content of the epididymal fat pad may be unchanged in the American variety of the obese hyperglycemic syndrome in spite of the marked reduction in the number of fat cells per unit volume of tissue.

The fundamental differences between the two categories of obesity represented by the AO and ANO mice have been found previously to be reflected in the total weights of the epididymal fat pads during adult age (HELLMAN et al. 1962 c). The presence of a large number of mast cells also in the adipose tissue of the mice with a hyperphagia induced by goldthioglucose suggests that this phenomenon is related more to state of obesity than to its basic cause. The present observation that non fat cells under certain circumstances may contribute very much to the total number of cells in the fat depots stresses the importance of including histological methods in studies of the regulatory mechanisms of this tissue. In such experiments, for example, the demonstration of an increased total amount of deoxyribonucleic acid may not necessarily reflect a corresponding increase in the number of fat cells (cf. ZINGG, ANGEL and STEINBERG 1962). Great interest has been attributed to the mast cells since they were first shown by JORPES (1946) to contain heparin. The physiological significance of the considerable accumulation of mast cells within the adipose tissue in the two types of obesity is difficult to assess. There is strong evidence for a local effect of the mast cells within the tissues. RILEY (1953) pointed out that though the mast cells often start life near the blood vessels they tend to move away from them with increasing maturity. During this process the mast cells slowly lose their granules and he concluded the released substances would appear to be destined for action within the tissues rather than within the blood stream. The multiplication of mast cells during infection and allergy represents another argument for a local cellular activity (BURKL 1952). Since heparin is thought to be released in combination with its albumin cofactor, the heparin complement according to SNELLMAN, SYLVÉN and JULÉN (1951), this big molecule would presumably have greater difficulties in leaving the tissue spaces for the blood (MACINTOSH 1956).

In discussing the possible local effects of the mast cells it must be borne in mind that in the rat in addition to heparin and histamine they also contain serotonin (WEST 1959, ERSPAMER 1961). With regard to the adipose tissue

seems however most relevant to focus the attention on heparin because of its specific role in the metabolism and transport of lipids The administration of heparin has been shown to activate or stimulate the production of a plasma compound possessing lipemia clearing properties (ANDERSSON and FAWCETT 1950 ROBINSON and FRENCH 1960) This lipemia clearing factor is now known to be a lipase which catalyses the hydrolysis of the triglyceride moiety of lipoproteins to fatty acids and glycerol (KORN 1955 a, b) It has been shown that heparin has not only the ability to release the enzyme *in vitro* from rat adipose tissue but also appears to be an essential component of the complete enzyme system (KORN 1957) The accumulation of mast cells in the adipose tissue in connection with obesity would be consistent with some previous observations in the rat, suggesting that the lipase activity of the adipose tissue is concerned with accumulation of fat in depots Thus a marked enzyme activity was noted in the fed rat, when the fat depots were being built up while it was low during fasting (HOLLENBERG 1959) Our attempts to demonstrate an *in vitro* effect of heparin on the lipid metabolism of the epididymal adipose tissue also in normal and obese hyperglycemic mice were however unsuccessful An influence of heparin on the FFA metabolism has been demonstrated in rat adipose tissue homogenates where the addition of heparin in low concentrations inhibited the FFA release (SALAMAN and ROBINSON, 1961) The negative results of the assay for clearing factor lipase activity in the incubation media with different heparin concentrations probably also reflect the existence of species differences in the *in vitro* response When for example heparin in amounts of 1—10 000 μg per ml was added to preparations of pig adipose tissue lipase there was no increase in the enzyme activity (LYNN and PERRYMAN 1960) and neither was the enzyme activated by heparin in extracts from chicken adipose tissue (KORN and QUIGLEY 1955 1957) That no effect of heparin was found with isolated epididymal adipose tissue from mice does not exclude however that the *in vivo* heparin secretion from mast cells might have an important local influence on the metabolic activity of the fat cells in obesity

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The Reflex Nature of the Physiological Adjustments to Diving and Their Afferent Pathway

By

HARALD T ANDERSEN[1]

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Abstract

ANDERSEN H T *The reflex nature of the physiological adjustments to diving and their afferent pathway* Acta physiol scand 1963 *58* 263—273 — The elicitation of the submersion apnoea and bradycardia has been investigated in intact and decerebrated ducks with and without various trigeminal lesions in order to study the reflex nature of the diving characteristics and to explore their afferent pathway It has been found that the circulatory as well as the respiratory adjustments to water immersion of the head are medullary reflexes entirely independent of higher levels of the central nervous system although the basic reflex of course may be influenced by activity in higher centers when the latters are intact The trigeminal nerve is the afferent pathway of the reflex arc by which the reactions to diving are induced upon submersion The ophthalmic division appears to be the most important branch of the V nerve in this respect but also the mandibular portion seems to be an element in the afferent limb of the reflex arc The maxillary nerve however could not be shown to serve such a function

The physiological adjustments which take place in diving reptiles birds and mammals upon experimental water immersion are well known But whereas the changes in respiratory circulatory and metabolic parameters have been extensively studied the nervous mechanisms controlling these reactions to submergence have received much less attention

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Huxley (1913a, b c) studied the reflex nature of the submersion apnoea the postural apnoea and the effects of these two phenomena on the heart rate of the duck She showed that the apnoeic response to water immersion is independent of higher levels of the central nervous system by demonstrating that apnoea was induced in decerebrates just as well as in intact individuals (1913a) She also reported that the heart rate of decerebrated ducks decreases during submergence (1913c) However, the experiments reported in this latter paper were carried out with the ducks in a position which is known to produce postural apnoea and retardation of the heart rate (Huxley 1913b c Noël Paton 1913), therefore it is not possible to decide whether or to which extent the posture may have influenced the heart rate Furthermore her results were not quite consistent because complete apnoea could not always be produced This she attributed to the fact that the experiments were performed on decerebrates shortly after the operation had been completed when the animals may still have been under influence of the anesthesia and the shock resulting from transection of the brainstem

Richet (1899) and Lombroso (1913) suggested that afferent impulses carried by the trigeminal nerve might be responsible for the elicitation of the physiological adjustments to diving and in a previous paper (Andersen 1963) it has been demonstrated that the circulatory modifications are elicited by the water immersion *per se* probably due to stimulation of certain receptors located in the region of the beak

The investigation reported in the present paper was undertaken in order to determine whether the circulatory adjustments to diving like the apnoea are dependent of higher levels of the central nervous system, adapting a method of submergence by which influence from the postural reflexes can be excluded with certainty (Andersen 1963) and to explore the afferent pathway of the diving reflexes assuming a trigeminal mediation of the proper impulses.

Material and Methods

General

Ten domestic ducks were used Detailed information about the treatment of the animals the arrangement of the diving experiments and the methods for recording heart rate and respiration have been given elsewhere (Andersen 1963) and will not be repeated here All of the ducks used in the series of experiments reported in the present paper were provided with tracheal cannulas which extended out of the water while the heads of the birds were submerged

Decerebration

Decerebrates were prepared under ether anesthesia by sucking out one or both cerebral hemispheres so that *tectum mesencephali* could be seen A blunt instrument was then pushed across and through the brainstem at the caudal part of the mesencephalon, through or just behind the optic lobes (*corpora bigemina*) At the time when the brainstem was sectioned the animal was kept as lightly anesthetized as possible The level of the

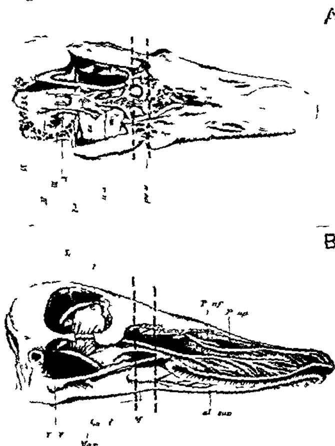


Fig 1 Level of section of trigeminal nerve supply to beak and nasal cavity between dotted lines (after E. CORDS 1904)

A. Dorsal view of head of goose Orbital roof r mov d

r.s. = *M rectus superior*
r.m. = *M rec'us med al s*
r l = *M r ctus lateralis*
o.s. = *M obl quus superio*
n. med = *Ram med alis nasi*

III *N ocul motor*
IV *N trochlearis*
V *N trigem nus*
VI = *N abd ens*

B Lateral v ew of head of duck showing ma n d vis ons of tr gem nal nerve Lateral part of beak removed

I = *N olfactorius*
V = *Ram op'thalmi us*
V + V = *Truncus maxillo-mand b laris*
Mand = *Ram mandibularis*
Max *Ram ma llar*

M *Ram mand b laris externus*
P nf *Ram praemax llaris i ferior*
P sup *Ram praema illaris superior*
alv up = *Ram alveolaris superior*

[1] The correspond ng structures of the duck are very s milarly arranged This illustrat on there fore shows the relat on of *am med al s nasi* to the various l ndmarks m ntioned n the text qu te satisfactorily

Table I Heart rate before during and after diving in intact (roman) *and decerebrated (italics) ducks*

Animal	Resting heart rate (beats/min)	Heart rate during submersion of head (beats/min)							Heart rate upon emergence (beats/min)			Duration of dive (min)
		15 sec	30 sec	1 min	2 min	3 min	4 min	5 min	15 sec	30 sec	1 min	
A	180	140	105	38	30	30	32	32	280	414	390	10
	138	*90*	*36*	*36*	*24*	*30*	*18*	*30*	*Not recorded*			*5*
B	222	125	40	30	24	24	24	—	400	37	375	4
	174	*38*	*30*	*30*	*24*	*30*	*30*	—	*200*	*294*	*192*	*4*
C	220	135	38	30	36	—	—	—	450	370	3 8	2
	150	*60*	*54*	*30*	*30*	—	—	—	[illegible]	*270*	*174*	[illegible]
D	204	152	122	72	48	48	—	—	180	260	260	3
	150	*120*	[illegible]	*45*	[illegible]	—	—	—	*225*	*310*	[illegible]	*2*
E	28	186	1 8	42	42	42	48	—	180	250	330	4.5
	2 0	*138*	[illegible]	*60*	*180*	—	—	—	*316*	*4 5*	*450*	*2.5*

section was confirmed by macroscopic inspection *post mortem*. Upon completion of the operative procedure all of the preparations displayed decerebrate rigidity. The latter persisted as long as the bird lived. The animals were allowed to recover for 1 hour after completion of the operation before any experiment was performed.

Trigeminal lesions

The distribution of the various branches of the trigeminal nerve has been studied in great detail in the goose and the duck by CORDS (1904). Her terminology will be used in this paper. The trigeminal branches supplying sensory innervation to the region of the beak, especially to the nostrils and the nasal cavity, were assumed to be of particular interest for the present study (ANDERSEN 1963). The nerve branches in question arise from all three main divisions of the trigeminal nerve. Nerves were cut under ether anesthesia in intact ducks or without anesthesia in decerebrates.

N. ophthalmicus. The nerve branches of ophthalmic origin innervating the beak and the nasal cavity are distributed from *ram. lateralis nasi*. They include the direct extension of the latter, *ram. medialis nasi* (Fig. 1A), and the two branches *ram. nasalis internus superior* and *ram. nasalis externus* which supply the caudal dorsal and the rostral part of the nasal cavity respectively. *Ram. nasalis externus* is also distributed to the nares and their immediate surroundings. The terminal branches of *ram. medialis nasi* are *ram. praemaxillaris inferior* and *ram. praemaxillaris superior*, the distribution of which are indicated in Fig. 1B. The level at which the ophthalmic nerve supply was severed is shown by the dotted lines in Fig. 1A. *Ram. medialis nasi* was reached by a dorsal approach. The skull was trephined at the border between *os frontale* and *os nasale* by which procedure the nerve can be seen at the ventral medial edge of the nasal bone. By exposing and cutting *ram. medialis nasi* at this level one also denervates the area, the sensory innervation of which is supplied by *ram. nasalis externus*, because the latter nerve has not yet left the main trunk. *Ram. nasalis internus superior* runs parallel and lateral to *ram. medialis nasi* and within the nasal bone where it may be reached and severed.

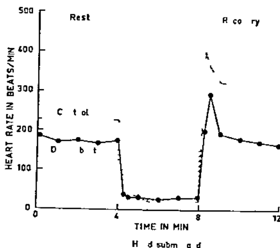


Fig 2 Heart rate before during and after immersion of head into water Before decerebration Dotted line After decerebration Solid line

N maxillaris This nerve is very easily exposed in the duck by a lateral skin incision midway between the eye and the caudal border of the beak It was always cut at this same level (Fig 1B) well caudal to the departure from the mother trunk of the branches distributed to the beak or the nasal cavity

N mandibularis The mandibular innervation to the beak was severed at the same level as the maxillary nerve (Fig 1B) *Ram mandibularis externus* is easily exposed and identified by a ventro-lateral incision through the skin The main trunk of the mandibular nerve however runs here as *ram circumflexus* through a channel in the bone of the lower jaw the lateral wall of which is very thin Therefore after having cut *ram mandibularis externus ram circumflexus* was exposed and sectioned by penetration of this wall.

Results

Decerebrates

Results obtained from diving experiments on decerebrated ducks are presented in Table I The experiment listed for subject B has also been illustrated in Fig 2 in order to provide a continuous record of the changes with time in the cardiac rhythm before during and after diving The data shows that the resting heart rate of the decerebrates was lower than that of the uninjured animals The initial rate of cardiac slowing upon water immersion of the head was about the same in controls and decerebrates as was the final level of the bradycardia during diving In one of the decerebrates subject E the heart rate started increasing while the head of the animal was still submerged However this preparation appeared to be faulty as judged by the fact that it lived for only 3 hours after decerebration whereas subjects A B and C lived for 10—12 hours All of the decerebrated ducks exhibited the abrupt post diving cardioacceleration which is usually displayed by a diving animal upon surfacing In all essentials therefore the responses of the cardiac rhythm to water immersion of the head were found to be the same before and after decerebration

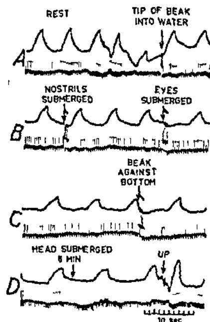


Fig 3 Respiration (upper tracing inspiration upwards) and heart rate (lower tracing) of decerebrate deprived of trigeminal innervation to beak and nasal cavity before during and after submersion of head
A B and C Continuous record Roughly 6 min between end of B and start of D

Decerebrates with trigeminal lesions

Upon water immersion of the head of a decerebrated duck in which all trigeminal branches to the region of the beak had been severed no respiratory or circulatory adjustments could be detected by the methods used This is shown in Fig 3 It appears that the bird continued breathing through its tracheal cannula during the descent and also while the head remained submerged for 6 min and the heart rate did not change appreciably before during or after diving Every experiment conducted on such preparations gave the same results

Effect of various trigeminal lesions

1 Complete denervation of the beak area Ducks with intact central nervous systems but with various deficits in the trigeminal innervation to the beak and the nasal cavity were also studied When the mandibular maxillary and ophthalmic supply to this region had been completely severed the usual responses to diving would as a rule not be elicited upon immersion of the head into water regardless of whether the duck could see or not A recording of the respiratory and cardiac activity of such a preparation before during and after diving might consequently appear virtually similar to that shown in Fig 3 for a decerebrate with corresponding trigeminal lesions However sooner or later the birds would act as if becoming aware of being submerged and respond by breath holding and struggling An example is furnished in Fig 4 In this

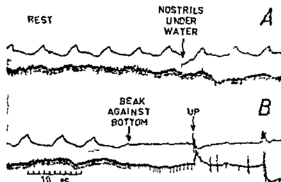


Fig 7 Respiration (upper tracing inspiration upwards) and heart rate (lower tracing) before during and after water immersion of head of duck. Ophthalmic innervation of beak and nasal cavity completely severed Blindfolded Duck continued diving for 20 sec after emersion
About 30 sec between A and B

When the nervous supply of ophthalmic origin had been completely severed by cutting the main branch of *ram medialis nasi* in addition to *ram nasalis internus* *prior* the same effects were obtained as those shown in Fig 6 provided the duck was blindfolded during the under water exposure This preparation did not require such careful handling during the descent as the one just previously described but also in this case would contact between the beak and the bottom of the water pool induce the diving characteristics (Fig 7) If the animal was pulled gently out of the water at this point it might continue diving for 15—30 sec. An example is furnished in Fig 7B

Finally if only the mandibular nerves were cut leaving the rest of the trigeminal innervation intact it was sometimes possible to immerse into water the part of the beak rostral to the nares without any change in respiration or heart rate However when the nostrils became submerged the diving characteristics usually appeared and upon immersion of the level of the eyes they were invariably elicited

Discussion

Although the term *diving reflexes* is occasionally encountered in the literature conservative authors hesitate to use it The cautious attitude towards the introduction of such a concept into physiological terminology is quite obviously due to our lack of knowledge about the reflex arc itself the nature of the sensory receptors in question and the adequate stimulus for their triggering

That physiological adjustments take place in response to water immersion of diving animals has been known for almost a century but during the ninety seven years which have elapsed since the publication of PAUL BERT's classical monograph (1870) physiologists have exclusively studied the diving characteristics proper and their efferent pathway whereas the afferent side of the ... has been entirely neglected RICHET (1899) working on di... the vagus nerve is the efferent limb of the reflex arc by ... absence of the reactions to diving upon submersion of

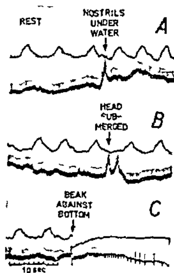


Fig 6 Respiration (upper tracing inspiration upwards) and heart rate (lower tracing) before and during descent Only lesion *Ram nasalis internus super* bilaterally severed. Blindfolded
A—C Continuous recording

II The importance of different trigeminal divisions An attempt was made to determine whether one of the main divisions of the trigeminal nerve is all important for inducing the physiological adjustments to diving or whether nervous information carried by all three of them contributes to the elicitation of the reactions to diving

When the maxillary nerve alone had been bilaterally sectioned the diving characteristics appeared as normally upon immersion of the head into water Conversely when the nerve supply of ophthalmic and mandibular origin had been severed leaving the maxillaries intact the duck behaved as if having been completely deprived of trigeminal innervation to the beak and the nasal cavity *i e* the diving reactions were not induced The recordings obtained in an experiment on the latter preparation are shown in Fig 5

The ophthalmic innervation of the beak and the nasal cavity is supplied by branches from *ram lateralis nasi* The caudal dorsal part of the nasal cavity may be denervated separately by bilateral section of *ram nasalis internus superior* This operation was performed in three animals and the respiratory and cardiac responses recorded from one of them while submerged blindfolded are shown in Fig 6 It appears that it was possible to avoid elicitation of the normal reactions to diving under such circumstances However the duck had to be submerged very carefully without audible splashes and if the beak was allowed to touch the bottom of the water pool apnoea and bradycardia resulted instantly (Fig 6C) Blindfolding was indispensible in order not to have the diving reactions elicited immediately upon contact between the beak and the surface of the water

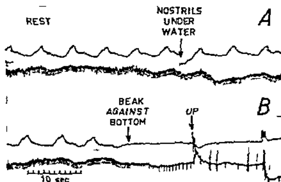


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nized birds His findings have later been confirmed in the alligator (ANDERSEN 1961), the snake (MURDAUGH and JACKSON 1962) and the seal (HARRISON 1960, MURDAUGH, SEABURY and MITCHELL 1961)

HUXLEY's studies (1913a) on decerebrated ducks showed that the apnoea elicited by water immersion of the head is independent of higher levels of the central nervous system, and the present investigation has shown that this is true also for the circulatory adjustments to submergence as determined by the appearance of marked bradycardia in response to immersion of the head of such preparations into water Taken together these findings exclude the necessity of cortical or thalamic integration of the sensory information inducing the diving characteristics and incorporate the diving reflexes into the group of medullary reflexes The question still remains however, whether the respiratory and circulatory adjustments are elicited independently, or whether the circulatory are secondary to the respiratory

The present investigation has also shown that the nervous impulses which give rise to the elicitation of the diving reflexes are mediated by the trigeminal nerve, more specifically, by the mandibular and especially the ophthalmic portions the maxillary nerve apparently being of little or no importance in this respect It may seem puzzling that nervous impulse traffic in branches of the ophthalmic nerve other than those innervating the nostrils and the nasal cavity as well as the mandibulary nerve should play a role in the elicitation of the diving characteristics however it is in perfect agreement with two findings previously reported (ANDERSEN 1963) When the head of a duck was introduced into a plastic cylinder which was subsequently filled with water the reactions to diving would be induced as usually and the bird would maintain the diving characteristics for 30—60 sec after the cylinder had been drained so that the nostrils were above the surface of the water while the rest of the beak remained submerged Likewise the respiration became slow and shallow, and the heart rate decreased markedly during descent already when only the part of the beak rostral to the nares was immersed into water The latter finding may, however, as well be interpreted as conditioning to, or anticipation of diving and there is indeed every reason to assume that the reactions to submergence are modified by activity at higher levels of the central nervous system when the latter is intact Such influence on the basic reflex is obvious from the experiments in which the sensory pathway had been partly interrupted (Figs 6 7) since it was necessary to blindfold the birds in order to make them continue normal respiration and cardiac activity during the period of under water exposure Likewise, cortical influence is apparent from the experiments conducted on ducks in which all trigeminal nerve supply to the beak areas had been severed (Fig 4) Here the duck endured water immersion of the head for 5 min before the respiratory efforts ceased and the bird began struggling vigorously and rhythmically It is interesting to observe the basic difference in diving behaviour of these two preparations When the diving characteristics were elicited in

the former for instance due to contact between the beak and the bottom of the water pool the duck displayed the quietness typical of a diving animal upon submersion The latter to the contrary appeared very uncomfortable if not panicky like a terrestrial animal upon becoming aware of being submerged It is tempting to speculate that the sensory messages from the beak region normally inhibits the respiratory center in the divers and that this is accomplished even if certain of the important sensory pathways are interrupted The rhythmic and violent struggling exhibited by the birds completely deprived of this sensory input to the central nervous system may consequently have been governed by the activity of the respiratory center the periodicity of which the struggling efforts paralleled closely Striking is also the absence of bradycardia under such circumstances

From the data presented in this paper it may be concluded that the circulatory responses to diving as well as the respiratory are independent of levels of the central nervous system higher than the medulla Furthermore our knowledge of the diving characteristics have been extended to include the afferent limb of the reflex arc The latter may be summarized as follows

Sensory receptors → trigeminal nerve → medullary centers → vagus nerve

In this scheme there is one point which ought to be worked out next namely the characteristics of the sensory receptors for as long as we are entirely without information about their nature and the adequate stimulus for their triggering the ultimate basis of the diving reflexes is still beyond our understanding

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Stimulation of Glucose Uptake in the Rat Diaphragm by Hydroxy-L-proline and L-lysine

By

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Abstract

BORREBÆK, B. and O. WALAAS. *Stimulation of glucose uptake in the rat diaphragm by hydroxy-L-proline and L-lysine.* Acta physiol. scand. 1963. 58. 274—284. — The influence of hydroxyproline, lysine and several other amino acids on glucose uptake of rat diaphragm *in vitro* has been investigated. Hydroxyproline increased glucose uptake and stimulated the incorporation of glucose C^{14} into glucose 6-phosphate of the diaphragm. The effect occurred at a concentration level of $1 \cdot 10^{-4}$ M—$5 \cdot 10^{-4}$ M hydroxyproline in the medium. At higher concentration of hydroxyproline, glucose uptake was decreased. In contrast to insulin, hydroxyproline did not promote glycogen synthesis and did not stimulate incorporation of glucose C^{14} into glycogen. When the diaphragm was incubated in a medium containing both hydroxyproline and insulin, the insulin effect on glucose uptake was depressed.

Lysine increased glucose uptake of the diaphragm, but less than hydroxyproline. No significant effect by any other single amino acids was found. However, a mixture of 13 natural amino acids, each at a concentration of $1 \cdot 10^{-4}$ M to $1 \cdot 10^{-3}$ M markedly depressed glucose uptake. The mechanism of a possible effect of hydroxyproline on the membrane carrier system for glucose uptake in muscle is discussed. Injection of hydroxyproline intraperitoneally *in vivo* exerted an hyperglycemic effect in the intact rat.

In a preliminary communication (WALAAS *et al.* 1958) it has been reported that hydroxy-L-proline and L-lysine at a concentration of 10^{-3} M stimulates glucose uptake by rat diaphragm *in vitro*. Recently BOLLINGER (1961) observed

that some other natural amino acids augmented glucose uptake of rat diaphragm while TELLER and BEIGELMAN (1961) found a depression of glucose uptake in rat epididymal fat tissue by several amino acids

The effect of hydroxyproline lysine and several other amino acids on glucose uptake of the diaphragm has been investigated in the present work Hydroxyproline was the most active amino acid in stimulating glucose uptake

Experimental

Male or female rats weighing 100—150 g which had been fasted 18—24 hours were decapitated and bled before removal of the diaphragm A pair of cut hemidiaphragms weighing 60—90 mg were used in each experiment The tissue was weighed and soaked in Krebs Ringer phosphate medium pH 7 4 containing D glucose at a concentration of 140 mg/100 ml for 20 min at 20 C The hemidiaphragms were thereupon blott d transferred to small Warburg vessels containing 1 ml of the same medium and incubated for 60 min at 37 C During the first 10 min of the incubation period the vessels were gassed with 100 / oxygen In some experiments incubation was performed in a Krebs-Ringer bicarbonate medium gassed with O_2/CO_2 (95 %/5 %) One hemidiaphragm acted as a control while the other half was incubated with the amino acid added to the medium Glucose uptake was determined by the glucose disappearance method with aid of glucose oxidase (HUGGET and NIXON 1957) The media containing amino acids were prepared by dissolving the amino acids in 100 ml of the standard medium to the desired concentration By measurement of pH of these media before and after incubation it was found that the change compared with the control media did not exceed 0 05—0 1 unit of pH

Preparation of glycogen from the diaphragms was done by the method of WALAAS and WALAAS (1950) and the glucose oxidase method used for the determination The initial value of glycogen was determined on one third of each hemidiaphragm which was removed prior to incubation

In experiments with C^{14} labeled glucose the diaphragms were soaked in Krebs-Ringer phosphate medium containing 215 mg/100 ml non radioactive glucose for 20 min at 20 C Incubation was performed in an identical medium to which had been added C^{14} labeled glucose diluted to a specific activity of 10 μc/mg After incubation for 4 min at 37 C the diaphragms were extracted with 1 ml 60 per cent ethanol for 30 min and centrifuged Reextraction with 1 ml 60 per cent ethanol was done twice The combined supernatants were purified by paper chromatography and high voltage ionophoresis on paper A complete description of this procedure has been given elsewhere (WALAAS *et al* 1963)

In vivo experiments were performed on male rats weighing 250 g After 24 hours fasting the rats were injected intraperitoneally with different concentrations of hydroxyproline dissolved in 0 5 ml Krebs Ringer bicarbonate medium pH 7 4 Blood samples were taken from the tail immediately before injection and at intervals during the first 3 hours after injection of hydroxyproline Control rats were injected with 0 5 ml Krebs Ringer bicarbonate medium Blood glucose was determined by the glucose oxidase method

The L-amino acids used were derived from Nutritional Biochemicals Corporation Cleveland Ohio Crystalline insulin was obtained from Allen and Handbury s Ltd and from British Drug Houses Ltd Glucose oxidase was a product of the Sigma Chemical Company

Table I The influence of hydroxyproline and lysine on glucose uptake of rat diaphragm. Experimental conditions described in the procedure

Amino acid added	Molar concentration	No of experiments	Glucose uptake			P[1]
			Control	With amino acid	Difference	
			mg/g wet tissue/hour			
L-hydroxyproline	5 10[illegible]	4	3.65	4.15	+ 0.50	< 0.001
L-hydroxyproline	1 10[illegible]	7	3.60	4.40	+ 0.80	< 0.001
L-hydroxyproline	5 10[illegible]	20	4.05	4.95	+ 0.90	< 0.001
L-hydroxyproline	1 10[illegible]	12	4.00	5.15	+ 1.15	< 0.001
L-hydroxyproline	1 10[illegible]	4	4.30	3.80	− 0.50	< 0.01
L-lysine	3 10[illegible]	4	2.70	2.85	+ 0.15	> 0.2
L-lysine	5 10[illegible]	20	3.25	3.80	+ 0.55	< 0.001
L-lysine	1 10[illegible]	16	2.80	3.50	+ 0.70	< 0.001
L-lysine	3 10[illegible]	8	2.75	2.60	− 0.15	> 0.5
L-lysine	5 10[illegible]	9	3.35	3.00	− 0.35	< 0.05
L-lysine	8 10[illegible]	3	3.30	2.65	− 0.65	—
Insulin	0.1 U/ml	4	3.55	4.85	+ 1.30	< 0.001
No addition	—	10	3.05	3.10	+ 0.05	—

[1] Significance of control groups versus amino acid groups.

Table II The effect of hydroxyproline on glycogen synthesis of rat diaphragm during incubation in a glucose containing medium Standard experimental conditions

No. of exp.	Glycogen synthesis			P[1]
	Control	1 10[illegible] M hydroxyproline	Difference	
	mg/g wet tissue/hour			
8	1.55	1.20	− 0.35	0.0[illegible]

[1] Significance of control group versus hydroxyproline group

Results

From Table I it is seen that hydroxyproline as well as lysine significantly stimulated glucose uptake by the diaphragm In accordance with our previous report maximal effect by these amino acids was obtained at a concentration of the amino acids of 1 10[illegible] M in the medium The effect of hydroxyproline was significant down to a concentration of 5 10[illegible] M while lysine was less active and the effect appeared in a more narrow concentration range between 1 10[illegible] M and 5 10[illegible] M At higher concentrations both these two amino

Table III The influence of hydroxyproline and insulin on the incorporation of glucose C^{14} into glucose 6 P of rat diaphragm

Test substance	No of exp	Total radioactivity			P
		Control	Test substance added	Difference	
		cpm/100 mg wet tissue			
5 10⁻ M hydroxyproline	4	130	278	+ 148	< 0 0?
5 10⁻ M hydroxyproline	5	193	136	− 57	< 0 01
0.1 unit insulin	5	210	372	+ 16?	< 0 02

[1] Hydroxyproline not present during the 20 min soaking period Incubation with hydroxyproline for 4 min

[2] Hydroxyproline present in the medium during the 20 min soaking period as well as during the 1 min incubation period

Insulin present in the medium during the 20 min soaking period as well as during the 1 min incubation period.

Significance of control groups versus hydroxyproline and insulin groups

Table IV The influence of hydroxyproline and insulin on the incorporation of glucose C^{14} into glycogen of rat diaphragm

Test substance	No of exp	Total radioactivity			Specific activity			P
		Control	Test substance added	Difference Per cent	Control	Test substance added	Difference	
		cpm/100 mg wet tissue			cpm/mg glycogen			
5 10⁻⁴ M hydroxyproline	5	1 980	2 330	+ 17	24 7	32 1	+ 7 5	~ 0 2
0 1 unit insulin	5	449	936	+ 108	3 8	8 6	+ 4 8	< 0 05

After an incubation period of 4 min

After incubation for 1 min

Significance of control groups versus hydroxyproline and insulin groups

acids exerted a slight inhibition of glucose uptake The stimulation of glucose uptake by hydroxyproline and by lysine could be demonstrated by incubation in phosphate as well as in bicarbonate medium The maximal stimulating effect exerted by hydroxyproline on glucose uptake is of the same order of magnitude as that obtained with insulin in our laboratory However, in contrast to the marked stimulating effect of insulin on glycogen synthesis in the diaphragm any such effect by hydroxyproline was absent As shown in Table II hydroxyproline at a concentration of 1 10⁻⁴ M slightly inhibited the synthesis of glycogen in comparison with the normal control

Table V The influence of hydroxyproline + insulin simultaneously added to the incubation medium on glucose uptake of rat diaphragm

Additions	No. of exp	Glucose uptake		
		Control	Test substance	Difference
		mg/g wet tissue/hour		
0.1 U/ml insulin	4	3.55	4.85	+1.30
$1 \cdot 10^{-3}$ M hydroxyproline	12	4.00	5.15	+1.15
$2 \cdot 10^{-4}$ M hydroxyproline + 0.1 U/ml insulin	2	2.80	3.60	+0.80
$6 \cdot 10^{-4}$ M hydroxyproline + 0.1 U/ml insulin[1]	4	3.65	4.05	+0.40
$1 \cdot 10^{-3}$ M hydroxyproline + 0.1 U/ml insulin	4	3.60	4.00	+0.40

[1] Statistical significance

hydroxyproline + insulin versus insulin $P < 0.01$

hydroxyproline + insulin, versus hydroxyproline $P < 0.01$

These findings were further corroborated in experiments where C^{14} labeled glucose had been added to the incubation medium. As shown in Table III hydroxyproline at a concentration of $5 \cdot 10^{-4}$ M increased the total radioactivity in glucose-6-phosphate which can be explained by an increased turnover of glucose in the hexokinase reaction. The same effect has been demonstrated by addition of insulin. Furthermore it has been observed that by preliminary soaking with hydroxyproline for 20 min at 20° C prior to incubation the incorporation of glucose C^{14} into G-6-P was inhibited. This may be explained by an accumulation of hydroxyproline in the diaphragm during the preincubation period reaching a level where an inhibitory effect on glucose uptake takes place. There was a difference between the effect of hydroxyproline and insulin on incorporation of C^{14} labeled glucose into glycogen (Table IV). *Insulin increased the total radioactivity as well as the specific* activity of glycogen. In contrast the mean values of these figures were only slightly increased by hydroxyproline and not at a significant level.

In further experiments the possibility of an additive effect of hydroxyproline and insulin on glucose uptake was investigated. Insulin at a concentration of 0.1 U/ml which gives maximal effect was added to the medium containing hydroxyproline at active concentrations. As shown in Table V no evidence of an additive effect was obtained. On the contrary the insulin effect was abolished and glucose uptake was somewhat lower than that observed when either hydroxyproline or insulin alone were present in the medium.

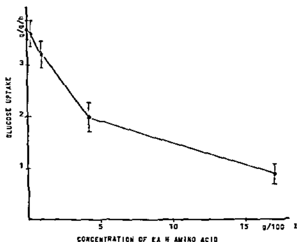


Fig 1 The inhibition of glucose uptake of rat diaphragm by a mixture of amino acids in the incubation medium The following amino acids were added L-alanine L-arginine L-cysteine L-histidine L-leucine L-isoleucine L lysine L-methionine L-phenylalanine L-threonine L-tryptophan L-tyrosine and L-valine Each amino acid was added at the concentration (mg/100 ml) shown on the abscissa The horizontal bars above and below each point indicate the standard deviations

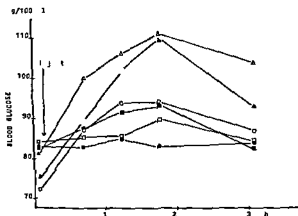


Fig 2 The influence of hydroxyproline injected intraperitoneally into rats on the blood glucose level

●——●	Controls injected with 0 5 ml Krebs Ringer bicarbonat
□——□	0 0023 mg hydroxyproline/g at injected
■——■	0 013 » » » »
○——○	0 045 » » » »
▲——▲	0 24 » » » »
△——△	0 46 » » » »

In similar experiments with mixtures of lysine and insulin the same tendency was observed However the differences were not as marked as with hydroxyproline and could not be considered as fully significant

In addition to hydroxyproline and lysine the influence of several other natural amino acids on glucose uptake were studied separately No significant stimulation was observed by the following single amino acids at a concentration of $1 \cdot 10^{-3}$ M L-alanine L-arginine, L-cysteine, L-glutamic acid, glycine L-histidine, L-leucine L-methionine L-proline L-serine L-tryptophan Some of these amino acids tended to give a slight although insignificant depression of glucose uptake Therefore, experiments were performed where the diaphragm was incubated in a medium containing a mixture of 13 amino acids each at concentrations of 0 25—15 mg/100 ml (Fig 1) In these experiments an inhibition of glucose uptake was observed with increasing concentration of the amino acids in the medium Thus when each single amino acid in the mixture was present at a concentration of 17 mg/100 ml (*i e* approximately $1 \cdot 10^{-3}$ M) the glucose uptake was reduced by 75 per cent

The possible effect of the hydroxyproline level in blood plasma on carbohydrate utilization *in vivo* was studied by determination of blood sugar after *injection of hydroxyproline in fasted rats It was observed that hydroxyproline* caused an increase of the blood sugar level (Fig 2) which reached a maximum 90—120 min after the injection This effect was obtained with rather high doses of hydroxyproline In experiments with smaller doses corresponding to the normal concentration range in plasma the hyperglycemic effect was absent However, hydroxyproline never produced hypoglycemia

Discussion

From the results reported it is apparent that the stimulating effect by hydroxyproline as well as by lysine on glucose uptake in the diaphragm occur at a certain concentration range of these amino acids in the incubation medium. *Hydroxyproline at a concentration of $1 \cdot 10^{-3}$ M has a maximal effect* but is still active at a concentration of $5 \cdot 10^{-4}$ M The stimulating effect of lysine occurs in the more narrow concentration interval of $5 \cdot 10^{-4}$ M to $1 \cdot 10^{-3}$ M At concentration above $1 \cdot 10^{-3}$ M hydroxyproline as well as lysine exert weak *inhibitory* effects Depression of glucose uptake by other amino acids has also been demonstrated by the inhibition which occurred when the medium contained a mixture of several amino acids at a total concentration above $1 \cdot 10^{-3}$ M

The somewhat different results reported by different workers concerning the effects of amino acids on glucose uptake can be explained by the observations reported above TELLER and BEIGELMAN (1961) observed an inhibition of glucose uptake in rat epididymal fat tissue by several amino acids such as arginine glutamic acid leucine isoleucine and lysine at concentrations of $1 \cdot 10^{-2}$ M—$1 \cdot 10^{-3}$ M This effect was diminished at lower concentrations In the work by BOLINGER (1961) hydroxyproline and lysine were *only tested at a concentration of* $1 \cdot 10^{-2}$ M where no stimulating effect can be expected

In contrast to our results a significant stimulation of glucose uptake by alanine leucine and isoleucine was observed This may be due to different strains and a different diet of the rats which may affect the influence of amino acid con centration on glucose uptake Undoubtedly it is necessary to test each individu al amino acid at several different concentrations such as has been done with hydroxyproline and lysine in the present work

MANCHESTER and YOUNG (1959) did not observe any inhibitory effect by mixtures of amino acid on glucose uptake such as has been reported here In their experiments the concentration of each amino acid was rather high 10 mg/100 ml The fact that methionine was omitted in their mixture can hardly explain the different results and we have no explanation for this dis crepancy

It now seems fairly well established that the most important rate limiting factor for glucose uptake into muscle is the transport of glucose across the cell membrane structure which is under hormonal control by insulin (LEVINE and GOLDSTEIN 1955, KIPNIS 1959, MORGAN *et al* 1959) However in spite of ex tensive work in this field very little is known of the mechanism of the mem brane transport and the action of insulin thereupon at the molecular level The report by KONO and COLOWICK (1961) that uncouplers such as 2 4 dinitrophenol and SH inhibitors such as p hydroxymercuribenzoate stimulate sugar membrane transport in the diaphragm is of interest in this connection

Hydroxyproline at the concentration range described in some respect has an insulin like effect on rat diaphragm The extent of the stimulating effect on glucose uptake as well as the increased incorporation of glucose C^{14} into glucose 6 phosphate corresponds to that observed with insulin Hydroxyproline probably acts on the glucose penetration mechanism as does insulin The ob servation that hydroxyproline depresses the stimulating action of insulin on glucose uptake supports the assumption that hydroxyproline competes with insulin for structural elements of the membrane transport system On the other hand, hydroxyproline has no stimulating effect on glycogen synthesis The same has been reported for other amino acids which can stimulate glucose uptake (BOLINGER 1961) According to LARNER VILLAR-PALASI and RICH MAN (1960) the stimulating effect of insulin on glycogen synthesis may be a specific insulin effect probably associated with an activation of the UDPG pathway (LARNER 1960) and distinct from the insulin effect on the glucose membrane transport mechanism

From the results of the present work a consideration of the role of hydroxy proline and particularly the significance of the OH group of this amino acid for glucose uptake in the diaphragm is of interest As shown in the experi mental part proline completely lacks the stimulating effect observed with hydroxyproline It is well known that hydroxyproline is almost exclusively a constituent of collagen which contains 13 per cent hydroxyproline Collagen is localized in the ground substance of connective and other tissues It is of

interest that KONO and COLOWICK (1961) have isolated membranlike structures from skeletal muscle containing collagen Thus, on a dry weight basis this membrane preparation contained 65 % protein, most of which was collagen

According to GUSTAVSON (1956), the OH groups in hydroxyproline play an important role in determining the stability and general reactivity of collagen GUSTAVSON (1958) has produced evidence for the formation of interchain hydrogen bonds between OH groups in hydroxyproline and the carbonyl oxygen of an adjacent helical peptide unit These types of bonds in the collagen of muscle membrane structures may be of importance for regulation of the membrane transport process This is in accordance with the hypothesis advanced by STEIN and DANIELLI (1956) that facilitated diffusion through living membranes may occur by aid of a hydrogen bonding component

The effect of hydroxyproline on sugar uptake of rat diaphragm cannot be explained by incorporation of hydroxyproline in the membrane collagen by a process of protein synthesis From experiments on intact animals it is known that isotopically labeled hydroxyproline is not incorporated into body proteins (STETTEN 1949) but rapidly metabolized in the liver (WOLF and LEAK 1956) From the work by GREEN and LOWTHER (1959) it is known that hydroxyproline is readily taken up by tissue slices and even concentrated within the cell By a mechanism similar to that described for collagen OH groups of hydroxyproline *taken up into muscle tissue may form hydrogen* bonds with membrane constituents and thereby influence the rate of sugar transport by a carrier complex

In seeking an explanation for the stimulating effect of lysine on glucose uptake in the diaphragm the possibility of the formation of complexes of glucose with lysine was investigated It is well known that the "browning reaction is due to interaction of α NH_2 and ε NH_2 groups of lysine with glucose (LEA and HANNAN 1950 GOTTSCHALK and PARTRIDGE 1950 MICHEEL and KLEMER 1956 FOLK 1956 LEWIN 1957) Terminal NH_2 groups of proteins also react with glucose (SCHWARTZ and LEA 1952 MICHEEL and KLEMER 1956) Therefore in some experiments the diaphragm was incubated in a medium containing lysine + radioactive glucose C^{14} and incubated as usual Subsequently the diaphragm was extracted with 60 per cent ethanol and the extract subjected to two-dimensional paper chromatography However the radioautographs were completely negative concerning the formation of radioactive labeled complexes in the presence of lysine Similar experiments with hydroxyproline also gave negative results

The observation of the stimulating effect by plasma dialysate on glucose uptake in the diaphragm (HAAVALDSEN and WALAAS 1957) confirmed by GJEDDE (1960) initiated the work here presented We have not been able however to ascribe this effect to the presence of hydroxyproline in plasma The concentration of plasma hydroxyproline is rather low (0 1 mg/100 ml in rat (WISS 1949) as well as in human blood plasma (OYE 1962) under fasting

conditions This corresponds to a concentration of hydroxyproline where no effect on glucose uptake could be expected *in vitro* The total amino acid concentration in blood plasma under fasting conditions amounts to 2[illegible]—30 mg/100 ml. In our *in vitro* experiments a mixture of amino acids at this total concentration gave slight inhibition of glucose uptake

When hydroxyproline was injected *in vivo* into rats, no evidence of any hypoglycemic effect by hydroxyproline was observed On the contrary injection of 0.045 mg hydroxyproline per g rat which by equilibration in the water space of the body increases the hydroxyproline level to 2 mg/100 ml (i.e. approximately $1 \cdot 10^{-4}$ M) produced hyperglycemia. This hyperglycemic effect may be due to metabolic conversion of hydroxyproline in the liver The conversion of hydroxyproline to aspartic and glutamic acid alanine (Wolf and Leak 1956) and glycogen (Hess and Shaffran 1951) can contribute to the formation of glucose-6-phosphate with increased sugar output. A second possibility is that the hyperglycemic effect is due to a depression of the insulin effect on the uptake of glucose in the peripheral tissue as has been shown *in vitro* in this work

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Acta physiol scand. 1963 58 285—286

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Histochemical Demonstration of Fluorogenic Amines in the Cytoplasm of Sympathetic Ganglion Cells of the Rat

By

OLAVI ERÄNKÖ and MATTI HÄRKÖNEN

Sympathetic ganglia are known to contain catecholamines predominantly noradrenaline (EULER 1947 VOGT 1954) However, little is known of the distribution of these amines within the ganglion In a study mainly dealing with the peripheral sympathetic structures FALCK (1962) briefly stated that a varying number of ganglion cells in adrenergic ganglia show a positive histochemical reaction for noradrenaline in the cytoplasm and in the larger processes We are not aware of any other study in this subject

Formalin induced fluorescence has long been used for histochemical demonstration of noradrenaline-containing chromaffin cells (ERÄNKÖ 1955) Only recently it was observed independently in 3 laboratories that dry formaldehyde vapour converts several amines into strongly fluorescent compounds whose distribution in frozen-dried sections can be studied by fluorescence microscopy (ERÄNKÖ 1961 1963 FALCK and TORP 1961 FALCK 1962 LAGUNOFF PHILLIPS and BENDITT 1961) The present report deals with the superior cervical ganglion of the rat in which noradrenaline can be expected to be the main fluorogenic amine Fresh ganglia were frozen and dried at — 40 C Freeze dried tissue was exposed to dry formaldehyde vapour before or after embedding in paraffin wax and sectioning (for details see ERÄNKÖ 1963)

A green fluorescence was observed in the cytoplasm of all ganglion cells and in nerve fibres between them (Fig 1) The fluorescence intensity varied from one cell to another ranging from weak to strong through intermediate stages A diffuse cytoplasmic fluorescence was seen in all nerve cells but in addition to it brilliantly fluorescent small granules were observed in the cytoplasm of many cells and in the fibres between the cells In cells whose axons were visible in the section the view suggested migration of the granules from the cytoplasm to the axon (Fig 2) Especially near bundles of nerve fibres occasional small cells exhibited an extremely bright yellow fluorescence These cells were very few and apparently they were not nerve cells Ganglia taken from rats whose catecholamine stores had been depleted with large doses of reserpine were essentially non fluorescent

The observations described suggest (1) that sympathetic ganglion cells manufacture and store catecholamines in the cytoplasm (2) that the catecholamine content greatly varies in individual cells (3) that a part of the

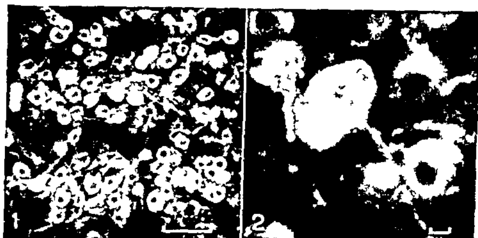


Fig 1 Formaldehyde induced fluorescence in the superior cervical gangl on of the rat Note great variations in the fluorescence intensity of individual cells Siemens lamp HBO 200 Sch tt filters BG 12 and OC 1 Scale mark 100 μ
Fig 2 Cells from the same specimen Brightly fluorescent granules are vi ible in the d ffusely fluorescent cytoplasm and along the axon originating from it Scale mark 10 μ

catecholamine is in a soluble form while another part is concentrated in granules and (4) that these granules migrate from the cytoplasm to the axon

These tentative conclusions which should be tested by other methods such as electron microscopy and analysis of cytoplasmic granule fractions fit well with the recent observations which indicate that noradrenaline is partly luble partly bound in granules in sympathetic nerves and in adrenergic nerve rminals (EULER and HILLARP 1956 FALCK 1962)

This study has been supported by grants from the S grid Ju élius Foundat on Helsinki and from the National Institute of Arthritis and Metabolic Diseases U S Public Health Service (A 1725)

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Acta physiol scand 1963 58 287—291

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Studies on SO, NO and NH_3 Effect on Ciliary Activity in Rabbit Trachea of Single in Vitro Exposure and Resorption in Rabbit Nasal Cavity

By

TORE DALHAMN and JAN SJOHOLM

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Abstract

DALHAMN T and J SJOHOLM *Studies on SO_2, NO_2 and NH_3 effect on ciliary activity in rabbit trachea of single in vitro exposure and resorption in rabbit nasal cavity* Acta physiol scand 1963 *58* 287—291 — One of the factors which determine the toxicity of pulmonary irritant gases etc presumably is their action on the ciliated epithelium of the respiratory tract. The degree to which such gases are resorbed in the mucous layer of the respiratory passages must also be taken into account when hygienic limits and allied questions are discussed The present paper illustrates these two factors viz ciliostatic action and resorption by experiments with three common respiratory irritant gases sulphur dioxide nitrogen dioxide and ammonia As regards concentration required to arrest tracheal ciliary activity *in vitro* the three gases varied considerably The degree of resorption in the upper respiratory tract also showed wide variations It seems probable that a gas which even in low concentration rapidly impairs ciliary activity and which is resorbed to a relatively slight degree can penetrate deeper into the bronchial tree and thus on the stated assumptions be more toxic than gases with the reverse characteristics

In judging the influence of gases on the ciliated epithelium of the respiratory tract knowledge of their resorption in the upper passages is highly important This resorption is dependent upon many chemical and physical factors which

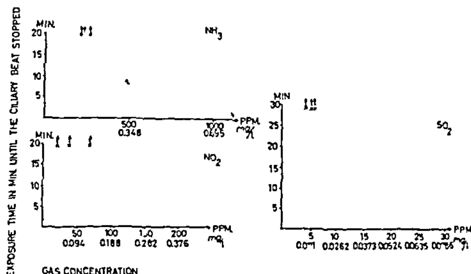


Fig 1 Effect of various concentrations of SO, NO, and NH on tracheal ciliary activity in vitro



Fig 2 Resorption of various concentrations of SO_2, NO and NH in rabbit nasal cavity

are outside the scope of the present paper As the secretion in the nasal cavity and bronchi contains approximately 3 per cent mucin 2 per cent salts and 95 per cent water presumably one of the decisive factors in resorption of gases is their solubility in water

In this paper some earlier observations concerning resorption of sulphur dioxide and ammonia are presented along with results of analogous experiments with nitrogen dioxide (DALHAMN and STRANDBERG 1961 DALHAMN 1962) Studies of the effect of NO_2 on tracheal ciliary activity are also reported together with corresponding earlier findings on SO_2 and NH_3

Table I Resorption of various concentrations of SO_2, NO_2 and NH_3 in rabbit nasal cavity

Rabbit no	Gas (mg/l)		Resorption (%) after		
			15 min	30 min	45 min
1	*SO*	104	96	96	96
2		106	96	95	95
3		119	97	97	97
4		119	95	91	88
5		121	97	97	95
6		161	98	96	96
7		178	94	96	96
8		180	98	98	96
9		200	98	98	97
10		206	95	94	93
11		290	92	92	95
12		293	93	91	91
13		299	92	91	87
14		304	95	96	91
15		318	85	87	85
1	*NO*	24	70	56	65
2		47	57	52	51
3		70	71	67	55
4		86	49	53	38
5		145	57	54	57
6		164	19	39	34
7		173	65	70	60
8		184	47	64	38
9		232	54	47	48
10		246	46	78	78
11		260	60	45	31
12		412	30	44	42
1	*NH_3*	2,203	—	—	94
2		2 910	—	—	96
3		1 925	—	—	95
4		2 020	—	—	93
5		1 938	—	—	96
6		2,286	—	—	95

A. *Effect of Single in Vitro Exposure to SO_2, NO_2 and NH_3 on Ciliary Activity in Rabbit Trachea*

Technique The excised trachea was opened by longitudinal incision and was placed in a chamber with 100 per cent relative humidity and temperature 37 C From a gas-mixing apparatus (DALHAMN 1956 and 1962) which supplied a constant concentration the gas and air mixture was conducted to a wash bottle containing a small amount of water The bottle stood in the chamber so that the gas mixture was warmed and moistened before it was blown through an outflow tube over the trachea at a rate of about one litre per minute The exposure time was 20 min for NH_3 and NO_2 and 30 min for SO_2 At each experiment the concentration of gas in the outflow tube was determined

The results are given in Fig. 1, where each dot represents one animal. This shows that each of the gases used requires widely varying concentrations to cause arrest of ciliary activity during for instance 5 min exposure. The required concentration was 500—1,000 mg/l for NH_3, 150—200 mg/l for NO_2 and 20—30 mg/l only for SO_2.

B. *Studies on the Resorptional Capacity of Rabbit Nasal Cavity with Respect to SO_2, NO_2 and NH_3*

Technique. The trachea of living anesthetized rabbit was exposed and was cut completely across. A glass tube was passed into the cranial portion of the trachea; there was no air leak between the cannula and the tracheal wall. The caudal end of the trachea was free. The rabbit's jaws were closed and its head was placed in a chamber with a constant concentration of gas. The gas mixture was sucked continuously through the cannula for 45 min at a rate of 1 litre per minute. Analyses of the gas that had passed through the nose were made after 15, 30 and 45 min in the experiments with SO_2 and NO_2. Analysis of NH_3 was made only after 45 min. This was because very high concentrations of NH_3 had to be used in the chamber before the gas became at all demonstrable after passage through the nose. For the same reason the experiments with NH_3 were mainly confined to one concentration region.

The results are given in Fig. 2 and in Table I. SO_2 and NH_3 show a strong tendency of resorption also at high concentrations. Considerably diverging is NO_2, which even at the lowest concentration, 24 mg/l, is resorbed to a lower degree than the other two gases at considerably higher concentrations. Furthermore it is remarkable that the resorption capacity of the mucosa does not show any obvious decrease during the 45 min exposure to SO_2 and NO_2.

Discussion and Summary

The effects of irritating gases and vapours on the respiratory epithelium can be manifold. One of the most important reactions would seem to be that of the influence of the ciliary activity. At any given concentration of gas, however, it is to be expected that the effect on the cilia will decrease as the gas penetrates down into the respiratory passages. A proportion will have been resorbed mainly in the nasal cavity. The toxic action of gases and vapours would thus seem to be partly dependent upon their resorption in the upper respiratory tract.

The method is comparatively simple and is not intended to give accurate quantitative results. Its main purpose is to demonstrate distinct differences between the relevant properties of different gases.

In their ability to cause arrest of ciliary activity after e. g. 5 min of exposure the 3 gases varied widely. The required approximate concentration was 500 to 1 000 mg/l for NH_3, 150 to 200 mg/l for NO_2 and 20 to 30 mg/l for SO_2. These results correspond well with those found by CRALLEY (1942). Using a similar

method he has found that NH_3 requires 500 mg/l NO_2 100 mg/l and SO_2 30 mg/l to stop the ciliary activity

In the studies on resorption it was desirable for technical reasons that at least 5—10 per cent of the original gas concentration should still be detectible after passage through the rabbit's nose It was therefore necessary to begin with low gas concentrations which were gradually increased until the 5—10 per cent or higher residue was obtained Each of these experiments was done on separate animals For example the concentration of NH_3 had to be raised to about 2 000 mg/l in order to avoid total resorption More than 90 per cent of this gas was resorbed in all experiments using concentrations around 2 000 to 3 000 mg/l SO_2 in concentrations of 100 to 300 mg/l was resorbed to the high level of 85 to 95 per cent In marked contrast to these high degrees of resorption were the values for NO_2 The approximate concentrations in the experiments were 20 to 400 mg/l Resorption was less (31 to 78 per cent) than for SO_2 and NH_3 even when the lowest initial concentrations were used

The resorptional capacity of the nose for SO_2 and NO_2 seemed to be almost uninfluenced by the time factor up to 45 min

It was thus demonstrated that the studied gases differed as to concentration at which ciliary activity was stopped They also differed with respect to resorption in the nasal cavity of rabbits

The observations do not permit precise conclusions concerning the toxicity of these gases It seems probable however that a gas which rapidly affects ciliary activity in low concentrations and is relatively little resorbed in the nasal cavity will be more toxic than a gas which is largely resorbed in the nose and has little influence on the cilia The method outlined in this paper may be suitable for coarse screening of gases and vapours concerning their effect on ciliary activity in the trachea and resorption in the nose

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The Permeability of Capillaries in Various Organs as Determined by Use of the 'Indicator Diffusion' Method

I

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Abstract

CRONE, C. *The permeability of capillaries in various organs as determined by use of the 'Indicator Diffusion' method.* Acta physiol. scand. 1963. 58. 292—305. — The theory of a single injection technique, the Indicator Diffusion method, for quantitative studies of capillary permeability is developed. It is shown that the permeability of a capillary area can be expressed by three parameters: the initial extraction (F) of test substances added in a single injection to the blood flowing to an organ, the blood flow (Q) and the surface area (A) of the capillaries. The equation relating these figures is $P = Q/A \log_e 1/(1-F)$. The permeability coefficients of capillaries in kidney, liver, lung, brain and hind limb to inulin and sucrose are reported. It is found that the permeability of capillaries varies considerably from organ to organ. It is questioned whether the pore model adequately describes the functional characteristics of the capillaries in the muscles. The existence of pores should result in a pronounced deviation of the ratio between the permeability coefficients for sucrose and inulin from the ratio between the free diffusion coefficients. This was not found to be the case.

One of the most important activities of the vascular system occurs in the capillaries. Yet our knowledge about the membrane across which the exchange between blood and interstitial fluid takes place is scanty. While many biological structures of very small size have been submitted to direct physiological study in later years, the capillaries have not yet yielded to a direct attack. Thus our knowledge of the permeability characteristics of the capillary wall has still to be deduced from experiments on a mass of capillaries. In the part

of capillary physiology dealing with permeability quantitative studies have been advanced considerably by Pappenheimer and his coworkers in the last decade (PAPPENHEIMER 1953 RENKIN and PAPPENHEIMER 1957) This article is concerned with such quantitative studies and gives data for the permeability of the capillaries to two polar non electrolytes (inulin and sucrose) in brain lung liver kidney and hind limb

Measurement of the permeability of capillaries in situ implies that the test substances come in contact with the capillary membrane In actual experiments difficulties arise from the fact that two processes occur almost simultaneously the test substances injected are diluted in the blood and diffusion occurs through the capillary wall An experimental principle by which this problem may be solved was developed by CHINARD VOSBURGH and ENNS (1955) and ANTHONISEN and CRONE (1956) These authors made a single injection into an afferent artery of an organ of a diffusible test substance together with a non diffusible reference substance which was assumed to remain in the capillaries Immediately after the injection blood was rapidly collected from the vein draining the organ. The concentration of the reference substance in the individual samples is a measure of the degree of dilution at any time The extent to which the concentration of the diffusible substance is less than the concentration of the reference substance is a measure of the loss of the test substance

The loss thus determined must bear some relation to the permeability of the capillaries It was for some time thought that the loss was a direct indicator of the permeability However this is not so for the loss is not proportional to the permeability coefficient of the capillaries to the test substance Nonetheless it is possible to derive a simple expression for the relation between permeability coefficient and the loss as shown below With this type of analysis the Indicator Diffusion method as the experimental approach will be called becomes useful for characterizing the physical processes of transcapillary exchange

Recent investigations with the electron microscope show pronounced morphological differences of the capillary walls in different organs (BENNETT LUFT and HAMPTON 1959 FLOREY 1961) This demonstration of their variability is borne out by the present work which gives evidence for differences in the permeability of capillaries in brain lung liver kidney and the hind limbs Inulin and sucrose were chosen as test substances because they are inert easy to determine do not penetrate the cells and are of appropriate molecular size (14 5 and 4 40 Å radius respectively)

Theory

Fig 1 shows a time-concentration curve of the type obtained in the experiments The curve for the concentration of the test substance (c_{in}) would be identical with that of the reference substance (c_{ref}) if there were no loss

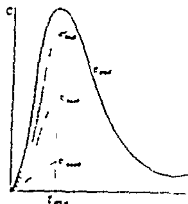


Fig. 1. The concentration variations in the effluent blood after a rapid square wave injection of a mixture of substances into the afferent vessel of an organ. c_{ref} = intravascular reference substance, c_{test} = diffusible test substances.

during passage through the capillaries. The curve c_{test} indicates the actual concentration of the test substance(s) and the fractional loss is given by $(c_{ref} - c_{test})/c_{ref}$. (As it is impracticable to inject equimolar quantities of reference and test substances the concentrations are expressed as fractions of those in the solution injected.) While the intravascular concentration is rising steeply the rate of outward diffusion is greatest. When the concentration is falling the net-diffusion decreases. The observations are made under conditions of continuous change and it is necessary to decide which samples can be used for estimating the average initial extraction. Only the rising part of the curve is considered in these calculations involving 3–5 samples from each experiment.

The initial extraction (E) of a test substance is not only dependent on the permeability of the capillaries but also on the surface area of the capillaries and on the rate of blood flow.

The permeability (P) of a membrane to a given substance is the amount of substance which passes unit area in unit time for unit concentration difference across the membrane. Or expressed in symbols

$$P = \frac{dS}{dt} \cdot \frac{1}{A \cdot \Delta c} \qquad (1)$$

dS/dt, the amount of substance passing the membrane in unit time, is determined as $Q \times E \times c$, where Q is the volume of blood passing through one gramme of tissue per second, E is the fractional reduction of the arterial concentration of the test substance during the conversion of arterial to venous blood. A is the surface of the capillaries in one gramme of tissue. Δc is the average concentration difference across the capillary wall.

The main difficulty in measuring the permeability of inaccessible biological membranes is the determination of Δc. An expression for Δc can, however, be derived if certain approximations are made. During the initial rapid rise of

intracapillary concentration that outside is increasing comparatively slowly The mean concentration difference (Δc) is therefore assumed to be equal to the mean intracapillary concentration The mean intracapillary concentration can be expressed in terms of the concentration of the test substance at the inlet c and the concentration of the test substance at the outlet c The test substance passes through the capillary wall passively and the concentration therefore falls exponentially from the arterial to the venous end of the capillary Thus the mean concentration is given by $(c - c)/(\log c - \log c)$ E is by definition equal to $(c - c)/c$ By inserting in equation (1) the following relation is obtained

$$P = \frac{Q}{A} \times \log \frac{1}{1 - E} \qquad (2)$$

By means of this expression it is now possible to calculate the capillary permeability from data obtained by the Indicator Diffusion method The term E is based on figures obtained from the experiments It is not practicable to measure the tissue perfusion Q because the experiments last less than a minute and therefore values for this term were selected from the publications of other workers The capillary surface area A was calculated from various anatomical measurements of capillary density in different tissues (see *Appendix* for further details)

Experimental Technique

The experiments were performed on mongrel dogs which were fasted overnight Anaesthesia was obtained by intravenous injection of Sodium Pentobarbitone (Nembutal) c 25 mg/kg A cannula was inserted into the afferent vessel of the organ concerned without interfering with the blood supply to the organ Another cannula fitted to a polythene catheter was introduced into the efferent vessel just before the injection The injection took 1—2 seconds and usually 2—5 ml of solution were used The out flowing blood was collected in small heparinized glass tubes mounted on a slowly moving Kymograph (ASMUSSEN and NIELSEN 1952) Usually 0 3—0 5 ml blood was collected in each sample The solution injected contained Evans Blue Dye in amounts between 2—5 mg depending on the dilution volume (greatest in experiments on lungs) A solution of inulin or sucros was mixed with the dye solution When the test substance was inulin sodium chloride was added to obtain isosmolarity An aliquot of the injection solution was diluted in heparinized blood taken from the animal immediately before the injection This mixture was later analyzed and thus the exact concentrations in the injection solution were determined Immediately after the experiment the small test tubes were stoppered with corks covered with paraffin wax and rotated gently The determinations of the concentration of the dye and of the test substances were carried out on blood rather than on plasma Thus it was unnecessary to use correction factors to account for material lost in the erythrocytes

Determination of concentration of dye and of test substances

a) *Evans Blue Dye* 0 2 ml blood was diluted with 0 8 ml 0 9 sodium chloride centrifuged and the concentration in the supernatant was determined by way of a

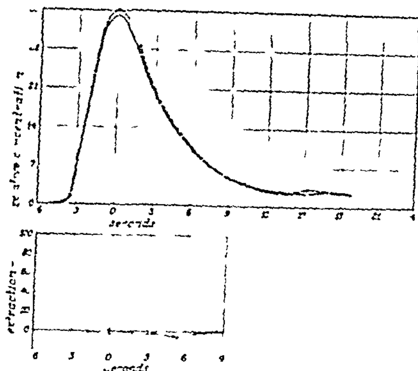


Fig. ? Inul n ... percent on k x n. The upper part of the figure shows time-concentration curves of E ans Blu Dye and of in 1 Sampling from the ... perior sagittal nu a er i jection into the common carotid artery. E ans Blue Dy ——— inulin ●———● The time for maximum concentration is arbitrarily called 0 time.
The lower part of the figure shows the extraction of inulin in separate samples from three experiments.

spectrophotometer (Beckman mod 1 DL. Semimicro cuvettes were used because the amounts of supernatant were small. The cuvettes were cleaned between each reading as the remaining fluid after emptying a semimicro cuvette is a large proportion of the total volume.

b) *Inulin and sucrose* were analyzed as described by Bojesen (195?). The volumes originally proposed by him were reduced to one tenth, and the hydrolysis and colour development took place in 2 ml glass ampoules the tips of which were sealed after introduction of reagent and sample. Inulin and sucrose added to blood in known amounts were recovered to 96.8 and 97.0 per cent respectively with standard deviations of 0.5(mg/100 ml and 0.40 mg/100 ml.

Calculation and treatment of data. All concentrations are expressed relatively, i.e. that in each sample is divided by that in the injection fluid. For each sample the relative concentration of the test substance is divided by that of the reference substance giving a value between 0 and 1. By subtracting this value from 1 the extraction of the test substance in that particular sample is obtained. The coefficient of variation of this final value is estimated to be less than 3

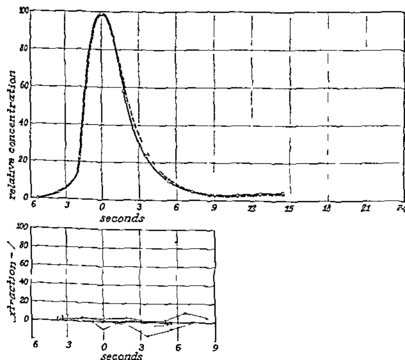


Fig 3 *Sucrose experiment on brain* The upper part of the curve shows time-concentration curves for Evans Blue Dye and for sucrose Evans Blue Dye ○——○ sucrose ●——● The lower part of the figure shows the extraction of sucrose in separate samples from four different experiments The point in brackets represents a sample in which the concentration of dye was very low (extinction < 0.100)

Results

1 *Brain*

Inulin The results of an experiment are given in fig 2 The curve for the test substance and the reference substance fall closely together indicating that the brain capillaries are highly impermeable to polysaccharides Three experiments were done and the extraction of inulin in each sample is shown in the lower part of Fig 2 The mean extraction was 2 5 % (S D 3 5 $n = 8$) In two experiments on human subjects a mean extraction of 0 5 % was found (the test solution was injected in the common carotid artery and cerebral venous blood was collected from a needle inserted in the bulb of the internal jugular vein)

Sucrose Similar results were obtained with sucrose which is also a polar molecule but much smaller than inulin Fig 3 shows that even such small molecules are not lost appreciably from the blood during the passage through the brain

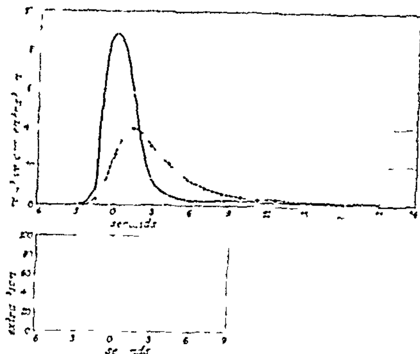


Fig 4 *Inulin permeation kidney* The upper part of the figure shows the concentration curves for Evans Blue Dye and Inulin Sampling from a catheter in the renal vein after injection to the renal artery Evans Blue Dye ——— Inulin ●——● The lower part of the figure shows the extraction of inulin in the separate samples

Fig 3 also shows the extraction in the individual samples The mean extraction in the initial phase was 0.4 % (S D 1.0 n = 8) One of the experiments was carried out in an animal which had sustained two periods of severe anoxia provoked by inhalation of pure nitrogen in 1.5 min The experiment was performed immediately after the second period of anoxia Contrary to what had been expected no change was observed in the extraction of sucrose Histological examination showed swollen chromatolytic ganglion cells in the cortex and in the basal ganglia These observations confirmed that the brain had been severely anoxic

2 *Kidney*

Ireis *et al* (1958) published an extensive series of investigations of the permeability of the renal capillaries to inulin Similar results were obtained in the experiment illustrated in Fig 4 The high initial loss of inulin is followed very quickly by a falling extraction and a net return occurs within a few seconds The mean extraction in the two initial samples was 73.1 % (the above mentioned authors found an average loss of 79 %)

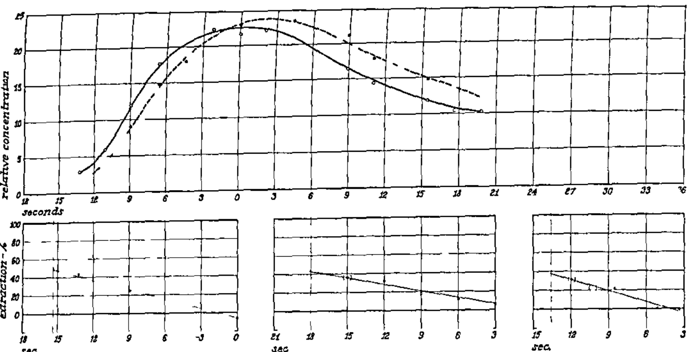


Fig 5 *Inulin experiment on liver* The upper part of the figure shows time concentration curves for Evans Blue Dye and inulin Sampling from a hepatic vein after injection into the portal vein Evans Blue Dye ○———○ inulin ● - - - ● The lower part of the figure shows the extraction in separate samples from three different experiments The extraction is falling on the rising part of the time concentration curve and the initial extraction is found by extrapolation (see text) The time for maximum concentration of dye is arbitrarily called 0 time The points in brackets represents samples in which the concentration of dye was very low (extinction < 0 100)

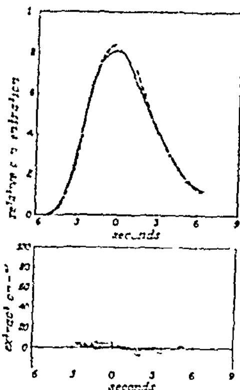


Fig. 4. *Ind[illegible] in leg* The upper part of the figure shows time-concentration curves for Evans Blue Dye and inulin. Evans Blue Dye ○——○, inulin ●——●. *The samples were collected from the femoral* artery after injection into the external jugular vein. The lower part of the figure shows the extraction of inulin in separate samples from six different experiments

The loss of inulin in the kidney results from loss through the glomerular membrane and through the peritubular capillaries. It is reasonable to assume that 20 % of the total material was lost in the glomerular capillaries by filtration. It is therefore likely that about 50 % of the inulin diffused through the peritubular capillaries.

3. *Liver*

Inulin. As might be expected the capillaries in the liver are very permeable to inulin. The experiments, however, show some peculiarities. Fig. 5 shows that the dilution curve is rather flat — an unexpected feature in view of the high rate of blood flow through the liver. This may be due to collection of blood from a long collecting catheter (which was introduced through the jugular vein into the hepatic veins) rather than a reflection of reduced blood flow during the experiment. It is also seen that the extraction of the test substance declines from the start. This means that the extraction cannot simply be calculated as an average from the early samples but must be found by extrapolation.

The narrowness of the extracellular space into which the test substance diffuses from the capillaries could account for the early decline in extraction

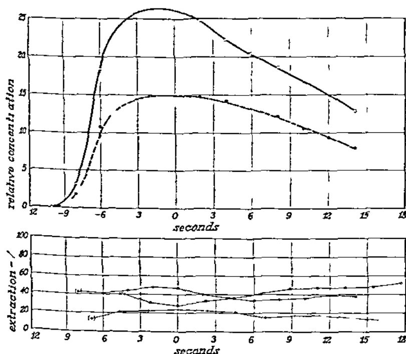


Fig 7 *Sucrose experiment on hind limb* The upper part of the figure shows time concentration curves for Evans Blue Dye and sucrose Evans Blue Dye ○——○ sucrose ●——● The lower part of the figure shows the extraction in separate samples from three different experiments. The points in brackets represent samples in which the extinction of Evans Blue Dye was below 0 100

If the extravascular distribution volume is very small an appreciable concentration builds up outside the capillary rapidly The space of Disse is extremely narrow which may account for the observations

In three experiments (shown on Fig 5) the average extraction of inulin was 43 3 %

Sucrose Comparable observations were made in three experiments with sucrose The average initial extraction was 72 3 %

4 *Lung*

Inulin The loss of inulin during its passage through the lungs was negligible as is shown on Fig 6 which summarizes the results of 6 experiments

Sucrose The lung capillaries were also impermeable to sucrose The high rate of blood flow reduces the fractional extraction of a substance from the blood passing through the lung capillaries It is obvious that the Indicator Diffusion method is inadequate for tissues with very high perfusion rates However it is possible to demonstrate a loss of certain substances from the

Table I [illegible]

Organ	Test substance	
	Inulin	Sucrose
Brain	0.0[illegible]	0.004
Kidney	0.73	—
Liver	0.43	0.[illegible]
Hind limb	0.11	0.33
Lung	0.0[illegible]	0.01

Table II [illegible]

Organ	Perfusion rate [illegible] 10⁴	Capillary surface cm² g	Permeability coefficients (cm sec⁻¹ 10⁵)	
			Inulin	Sucrose
Brain	9.0	40	0	0
Kidney	[illegible] 0	3.0	14.4	—
Liver	15.0	[illegible]0	[illegible]9	6.6
Lung	50.0	[illegible]0	0	0
Hind limb	1	0	0.[illegible]	0.[illegible]

lung capillaries Thus ANTHONISEN and CRONE (1956) found a high initial extraction of ethanol and CHINARD *et al* (1955) of D_2O and THO

Hind limb

Inulin The mean initial extraction was 10.7 % (S D 6.6 n = 21) While a decline in extraction was found in experiments on other organs in the hind limb it remained constant for a long period i e for at least 30 sec This may be explained by the functionally large extravascular distribution space in a resting muscle many capillaries do not function simultaneously so that the mean intercapillary distance is very high

Sucrose Fig 7 shows the results from experiments with sucrose The average extraction was 33.1 % (S D 11.5 n 9) The lower half of the figure shows that the extraction remains constant throughout the period of observation While in the liver the flatness of the time concentration curve was attributed to catheter artefacts the broad curve in the hind limb experiments is obviously due to the low rate of blood flow through resting muscle

As mentioned earlier one of the purposes of this work has been to characterize the capillary wall in terms of permeability coefficients The application of formula (2) to the average extractions tabulated in Table I gives the figures shown in Table II

Discussion

Although previous workers have found variations in the permeability of capillaries in different organs such observations have never been quantitative The Indicator Diffusion method depends on relationships shown in equation (2) the usefulness of which is still limited by lack of precise knowledge of the effective capillary surface area However it is reasonable to assume that errors involved are not large enough to invalidate the main conclusions drawn here

PAPPENHEIMER (1953) and RENKIN and PAPPENHEIMER (1957) have published the only data comparable with those given in this paper They have however been criticised for their use of van t Hoff s relation to determine the mean concentration difference across the capillary wall Δc (USSING 1953 GRIM 1953 KEDEM and KATCHALSKY 1957) The van t Hoff relation is only applicable to systems separated by a perfect semipermeable membrane or stated in STAVERMAN s terminology (1951) in systems where the reflection coefficient is 1 for all test molecules As the capillary membrane is permeable to both inulin and sucrose corrections to PAPPENHEIMER s figures have to be introduced KEDEM and KATCHALSKY give the values for the correction factors as 0 375 (inulin) and 0 058 (sucrose) With these correction factors the permeability coefficients found by PAPPENHEIMER become $0\,18 \times 10^{-5}$ cm sec^{-1} for inulin and $0\,30 \times 10^{-5}$ cm sec^{-1} for sucrose (hind limb capillaries of the cat)

The agreement between these corrected figures and the figures given in table 2 for the permeability of the hind limb capillaries in the dog is interesting considering that the results have been obtained with two fundamentally different experimental approaches based on different analyses of the problem The relation between the permeability coefficients is seen to differ only slightly from that of the diffusion coefficients The logical consequence of this finding is that the theory of restricted diffusion originally advanced by PAPPENHEIMER (1953) must be reconsidered (CRONE 1963)

RENKIN (1959) derived an expression for tissue permeability formally analogous to equation (2) No direct comparison can however be made between Renkin s figures and those presented here because of conceptual differences in the meaning of extraction in Renkin s deduction and in the present analysis Renkin determines the extraction in the steady state where the concentration of test substance outside the capillary wall is not necessarily insignificant With the Indicator Diffusion method determinations are made in a non steady state and extracapillary concentrations are thus of much less importance This means that the Indicator Diffusion method gives true capillary permeability

The finding that the brain capillaries are impermeable to inulin and sucrose raises the interesting question of how the brain is able to take up glucose from the blood in sufficient quantities The difference in molecular

diameter between sucrose and glucose is only a few Ångström Units. The chemical structure of both molecules is similar, both being highly polar with great tendency to form hydrogen bonds with the water molecules in the surroundings. These considerations lead to the conclusion (Crone 1960) that a special transfer mechanism must exist in the brain capillaries by means of which the passage of glucose into the brain tissue is facilitated. Experimental proof of this deduction will be given in a later paper.

Appendix

Determination of capillary surface area from anatomical data

1. *Brain.* According to Krogh (1929) the capillary length in 1 mm³ of grey and white matter is 1100—1400 mm and 300—400 mm respectively (rat brain). Craigie (1933), who also worked with brains from rats, found a length of about 1000—1100 mm in the cortex. Cobb (1932) found 1000 mm as the average length in cortex of man. As the blood samples in the present work contained mainly cortical blood, 1 : 1000 mm capillary length per mm³ was taken as a reasonable average figure. This gives a capillary surface of 240 cm²/g tissue.

2. *Muscle.* Krogh (1929) found a capillary surface of 5.0 cm²/cm³ resting muscle in a dog. This figure was authoritatively criticised by Renkin and Pappenheimer (1957) as being far too high. It would mean a capillary blood volume of 10 [illegible] of the total volume of the muscles. They found [illegible]0 cm²/g muscle from capillary counts on frozen sections. This latter figure was used in the present work.

3. *Lung.* Rossier (1956) calculated that the surface area of the capillaries of the lung in man was 33 m², which means that the capillary surface per g of tissue is [illegible]0 cm², assuming a lung weight of 1000 g.

4. *Kidney.* In a letter to the author professor [illegible] kindly informed me that he had found a total surface area of 3.49 m² of the peritubular capillaries in a dog kidney (weight 35 g). As the cortex weight is approximately [illegible]0 % of the total kidney tissue mass then the capillary surface area in the cortex is about 1.0 cm²/g (technique: [illegible] and Otto 19[illegible]9).

5. *Liver.* No data could be found concerning the surface area of these capillaries. By analogy with the values from other parenchymatous organs a tentative value of [illegible]0 cm²/g was used in the present work.

P. Eccleton gives estimates of various capillary surfaces in a chapter in Frey-Wyssling's book "Deformation and Flow in biological systems" (19[illegible]). The following values were given: Brain 240 cm²/g, Muscle [illegible] cm²/g, Kidney 134 cm²/g and Liver 133 cm²/g. The order of magnitude of these figures is seen to correspond quite well with those given above but no information of how the author obtained these figures is given.

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The Heparin Co-Factor Activity in Plasma and its Relation to the Anticoagulant Effect of Intravenously Injected Heparin

By

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Received 3 December 1[illegible]

Abstract

Blombäck, B., M. Blombäck, H. Lagergren and P. Olsson. *The heparin co-factor activity in plasma and its relation to the anticoagulant effect of intravenously injected heparin.* Acta physiol. scand. 1963. 58. 306—318. — The heparin co-factor activity in plasma, defined as the antithrombin activity at high heparin concentrations, was measured with two thrombin titration methods in dogs and human subjects following single or repeated intravenous injections of heparin. The co-factor activity was found to decrease continuously during the period of elimination of heparin from the plasma, after which it rose slowly to the initial level. In dogs the anticoagulant effect of intravenous heparin was studied at different levels of co-factor activity in the plasma. The co-factor activity was increased by infusion of bovine plasma fraction IV—1 prepared according to Cohn. The rise in co-factor activity was always accompanied by an increase in heparin effect (prolongation of the coagulation time). The co-factor alone exerted no effect on the coagulation time.

It was shown in a previous study (Olsson 1963) that the anticoagulant effect of i.v. injected heparin was probably not dependent on the plasma heparin level alone. Thus, the prolongation of coagulation time after an injection of heparin decreased much more rapidly than was implied by the heparin content of the blood. Moreover, a repeated heparin dose had a considerably

smaller anticoagulant effect than the first one and a higher dose exerted relatively less effect than a lower one It was suggested that these phenomena might be related to changes in the concentration of heparin co-factor in the blood

The object of the present investigation was to study the influence of heparin on the co-factor activity in plasma and also the anticoagulant effect of injected heparin at different co factor levels The co-factor activity in plasma was measured by two different methods in dogs and in human subjects both during and after elimination of i v injected heparin The anticoagulant effect of i v heparin was studied in dogs with a low and high heparin co factor activity in the plasma respectively

Material

1 *Fibrinogen* was prepared according to BLOMBACK and BLOMBACK (1956) The coagulability was 98—100 % when assayed by an ultraviolet spectrophotometric method (BLOMBACK 1958) The preparations were dissolved in 0 3 M NaCl to a protein concentration of 1 %

2 *Thrombin (bovine)* Prothrombin was prepared essentially according to SEEGERS LOOMIS and VANDENBELT (1945) In order to obtain a higher yield in conversion with thromboplastin and calcium ions the last purification steps following elution from $Mg(OH)_2$ were omitted The thrombin was further purified as described by BLOMBACK and YAMASHINA (1958) The activity was usually between 130 and 250 NIH units per mg of protein The thrombin was dissolved to the desired concentration in distilled water All vessels containing thrombin were siliconized When stored in siliconized vessels in ice water the solutions were stable for at least 5 hours

3 *Heparin* Commercial heparin in 5 solution (Vitrum Comp Stockholm) containing about 130 I U /mg

4 *Buffer* Tris (hydroxymethyl) amino-methane buffer pH 7 2—7 3 ionic strength 0 15

5 *Human and canine standard plasma* Citrated plasma from 6 individuals (1 part 3 8 Na citrate $\times 2H_2O$ + 9 parts blood) was mixed and centrifuged at 2 000 g for 20 min and the plasma pipetted off

6 *The plasma to be tested* was treated in the same way as the standard plasma The samples were always kept deep frozen until analysis could be performed

7 *Plasma fractions* Bovine plasma was fractionated by Cohn s method 6 (COHN *et al* 1946) Freshly prepared fraction IV—1 dissolved in saline (1/10 of the initial volume of the plasma) was used in most experiments The preparations had a heparin co-factor activity 10 to 20 times higher than that of normal dog plasma In other experiments fraction IV—1 was heated to 61 C for 10 min and the precipitate formed removed by centrifugation and discarded The volume was then adjusted to the original one with physiologic saline The heated fraction had no co-factor activity

8 *Experimental subjects* Both dogs and human subjects were used The weight of the former ranged from 11—16 kg When necessary the dogs were anesthetized by intraperitoneal injection of Nembutal© (Abbott) in a dose of 10 mg kg of body weight In these animals the samples were withdrawn through a polyethylene catheter (PF 190 introduced into the inferior vena cava via a peripheral vein in a hind leg and the injections made through a short catheter in a foreleg In unanesthetized dogs, heparin was administered and blood samples taken by direct venipuncture — The human subjects were healthy medical students Both blood sampling and injection were done by direct venipuncture

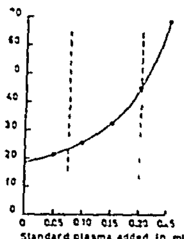


Fig. 1. Typical standard curve for determination of co-factor activity by the one-stage method. The clotting time of the test dilution is usually adjusted to fall between the dotted lines.

Methods

1. *Determination of heparin activity*

This was performed in plasma by the thrombin titration method of BLOMBÄCK *et al* (1953).

2. *Determination of heparin co-factor activity. Method 1. Quantitative two-stage thrombin titration*

The method has been described in detail elsewhere (BLOMBÄCK, BLOMBÄCK and OLSSON 1963). The test plasma was diluted 1/10 in tris buffer and the test performed at a heparin concentration of 4 I.U./ml of test mixture.

3. *Determination of heparin co-factor activity. — Method 2. One stage thrombin-time determination*[1]

Reagents

Fibrinogen-heparin solution. The fibrinogen stock solution was diluted with 0.15 M tris buffer to a protein concentration of 0.6%. The heparin stock solution was diluted in distilled water to a strength of 200 I.U./ml. Five ml of the heparin solution was added to 100 ml of the fibrinogen solution.

Thrombin solution. The stock solution was diluted in distilled water to 100 NIH units/ml.

Standard plasma. See "Material".

Procedure

0.50 ml of saline was pipetted into one test tube. 0.05, 0.10, 0.15, 0.20 and 0.25 ml respectively of standard plasma was pipetted into each of another 5 test tubes. In these 5 tubes the volume was adjusted to 0.5 ml with saline. One ml of fibrinogen-heparin solution was added to each tube. After 5 min incubation at 30° C, 0.5 ml of thrombin solution was added. A stop-watch was started at the moment of addition

[1] This method was devised by one of us (M. B.) in 1954, but since it has not been published earlier, it is outlined here.

of thrombin and the time when the first fibrin threads were caught by a platinum wire loop moved up and down in the tube was noted Double and triple determinations were made and the average value calculated The clotting time in the tube with saline only was adjusted by altering the strength of the thrombin so that it produced clotting in about 18 sec In the tubes containing plasma the clotting time increased The correlation between standard plasma concentration and coagulation time is shown in Fig 1 A new standard curve was plotted for each series The test sample was diluted in saline The clotting time of the test dilution should be within the steep part of the standard curve (Fig 1) Twelve samples with a co factor activity ranging from 123 to 25 % that of standard plasma were analyzed on two different standard curves on two separate days the standard deviation of a single determination was found to be 2 4 %

4 *Determination of coagulation time*

The samples for determination of the coagulation time were drawn from the caval catheter In most experiments the determination was made in glass tubes After discarding the first 2 ml 0 5 ml of blood was transferred with a 5 ml syringe into each of two tubes (9 × 75 mm) which were sealed and placed in a water bath at 30 C The tubes were gently tilted and rotated every 5 min The end point of coagulation was taken as the first appearance of fibrin threads and the average time for the two tubes was noted Only when the coagulation time was markedly prolong d i e was more than 20 min did the difference between the two tubes exceed 5 min The greatest difference recorded was 15 min this occurred on two occasions when the average time was 32 5 and 72 5 min respectively A few determinations of the coagulation time were made in a chamber according to JORPES BLOMBACK and BLOMBACK (1954)

Experimental and results

A Variation in co factor activity in plasma after i v injection of heparin

Four unanesthetized dogs were given a single injection of 800 I U /kg of body weight of heparin and four dogs 200 I U /kg Two control dogs received no heparin but were treated in the same way in all other respects Three human subjects were given a single injection of heparin in a dose of 400 I U /kg of body weight In every case a sample was taken before injection and 4 8 12 and 48 hours after it In most of the dogs a sample was also taken 72 hours after injection The heparin activity was determined as well as the heparin co-factor activity in plasma In these experiments the co factor activity was analysed both with the two stage quantitative thrombin titration method and with the one stage method

The effect of repeated heparin injections was studied in two dogs One dog was given 400 I U /kg and the other 800 I U /kg altogether 6 times in the course of 3 days The co-factor activity was determined during a 6-day period In these and all subsequent experiments only the one stage method was used

Three dogs under general anesthesia were injected with 800 I U of heparin/kg of body weight The heparin activity and the co-factor activity in plasma were measured before injection 30 and 60 min after it and then every 60 min for 5 hours In 3 anesthetized controls (no heparin injected) the co-factor activity was measured at 0 120 and 240 min

Table I. The changes of the co-factor activity in plasma in dogs following injection of heparin

Heparin dose (IU/kg body weight)	Time (hrs) after inj.	Heparin concentration (IU/ml plasma)	Thrombin (NIH units) inactivated by co-factor in 0.2 ml plasma[1]		Co-factor activity (% of activity in standard plasma)[2]	
		Mean	Mean	% of initial value	Mean	% of initial value
800 (Four dogs)	0	0.2	5.2	100	104.0	100
	4	1.04	4.6	89	[illegible]	[illegible]
	8	0.33	[illegible]	[illegible]	[illegible]	83
	12	0.29	1.9	37	[illegible]	[illegible]
	24	[illegible]	[illegible]	48	[illegible]	89
	48	[illegible]	—	—	[illegible]	93
	72	0.2	4.3	83	101.8	93
200 (Four dogs)	0	0.34	5.4	100	111.8	100
	4	[illegible]	4.0	[illegible]	[illegible]	79
	8	0.33	4.3	79	93.7	84
	12	0.34	4.9	91	102.0	91
	24	0.34	4.8	89	103.7	93
	48	0.30	4.9	91	[illegible]	[illegible]
	72	0.30	[illegible]	[illegible]	110.0	93
Control (Two dogs)	0	0.30	[illegible]	100	[illegible]	100
	4	[illegible]	7.5	108	109.0	104
	8	0.29	[illegible]	108	[illegible]	103
	12	0.29	7.5	103	[illegible]	102
	24	0.30	8.0	[illegible]	[illegible]	94
	48	[illegible]	7.5	109	[illegible]	101
	72	[illegible]	[illegible]	104	111.0	106

[1] Two-stage method.
[2] One-stage method.

The results of experiments on the unanesthetized dogs are listed in Table I. In the animals given a heparin dose of 800 I.U./kg. the thrombin inactivation (co-factor activity) in 0.2 ml of plasma, as measured by the two-stage method, decreased from an average of 5.2 NIH units of thrombin before injection to an average of 1.9 NIH units 12 hours after injection. Twenty four hours after heparin injection, the co-factor activity had started to return to the initial level. In these dogs, the heparin level was still elevated 4 hours after injection but 8 hours after, it had fallen to approximately the initial level.

In the 200 I.U. group, the decrease in heparin co-factor activity in plasma was less pronounced. Thus the thrombin inactivation fell from an initial of 5.4 NIH units of thrombin to 4.0 NIH units 4 hours after injection. At 8 hours

Table II The average changes of the heparin co factor activity in plasma in three humans following intravenous heparin 400 IU/kg bodyweight

Time (hrs) after inj	Heparin concentration (IU/ml plasma)	Thrombin (NIH units) inactivated by co-factor in 0.2 ml plasma[1]		Co-factor (of activity in standard plasma)	
	Mean of three	Mean of three	of initial value	Mean of three	of initial value
0	0	8.7	100	102.8	100
4	1.48	7.5	86	89.7	87
8	0.50	7.3	84	90.3	87
12	0.23	6.9	79	87.8	85
24	0.07	7.1	82	87.6	85
48	0	6.2	83	87.7	87

[1] Two-stage method.
[2] One stage method.

Table III Changes of the heparin co factor activity in plasma in dogs at repeated intravenous heparin injections

Time (hrs)	Heparin (400 IU/kg bodyweight)	Co-factor ( )	Heparin (800 IU/kg bodyweight)	Co-factor ( )
0	+	100	+	100
16	+	85.7	+	97.0
24	+	81.9	+	86.8
40	+	81.9	+	76.8
48	+	69.8	+	73.0
64	+	64.2	−	57.9
72	−	71.2	−	68.2
96	−	81.1	−	90.6
120	−	90.6	−	98.1
148	−	92.5	−	112.0

it had started to rise again The heparin concentration in plasma was slightly elevated 4 hours after injection as compared to the initial value In the dogs not given heparin no measurable changes in co-factor activity were recorded during the corresponding period

When the heparin co-factor activity in plasma was measured by the one stage method the average initial value was 104 % of that in standard plasma The changes in co-factor activity recorded with this method followed the same pattern as those obtained with the two-stage method When expressed as a percentage of the initial value the changes were however less pronounced when assayed by the one stage method

Table II. Changes of the heparin co-factor activity in plasma in dogs during general anaesthesia with a barbiturate and intravenous heparin

Time (min) after injection	Heparin (400 I.U./kg body weight)			No heparin	
	Heparin concentration (I.U./ml plasma)	Co-factor activity (% of activity in standard plasma)		Co-factor activity (% of activity in standard plasma)	
	Mean of four	Mean of four	% of initial value	Mean of three	% of initial value
0	0.7	107	100	10[illegible].8	100
30	1	9[illegible] 1	89	—	—
60	5 1	[illegible] 1	[illegible]	—	—
120	3 0	[illegible]	81	103 4	93
180	1	84 3	[illegible]		—
240	1 1	79 3	[illegible]	10[illegible] 1	[illegible]
300	0	80	[illegible]		—

One-stage method

In the 3 human subjects given a heparin injection of 400 I.U./kg the changes were essentially the same as in the dogs (Table II).

After repeated heparin injections into dogs a continuous decrease occurred in the heparin co-factor activity in plasma during the period of administration. After withdrawal of heparin the co-factor level started to rise (Table III).

The experiments on anaesthetized animals disclosed that the co-factor activity had started to decrease as soon as 30 min after heparin injection and continued during the time of heparin elimination (Table IV).

B. Anticoagulant effect of i.v. injected heparin in dogs with varying co-factor activity in the plasma

A decrease in the co-factor activity in the plasma was induced by i.v. injection of heparin in a dose ranging from 1 600—2 400 I.U./kg of body weight on the day before the actual experiment. An increase in co-factor activity was produced by infusion (during about 3 min) of bovine plasma fraction IV—1 in a dose ranging from 6—15 ml/kg of body weight. In controls corresponding doses of heated fraction IV—1 were infused.

Six experiments were made on dogs whose plasma co-factor activity had been reduced as just described. Four of these dogs were given an initial heparin injection of 400 I.U./kg of body weight. In two of them fraction IV—1 was injected 50 min later, in the other two heated fraction IV—1 was injected 15 and 65 min respectively after the heparin. The remaining two dogs were first given fraction IV—1 followed after 25 min by injection of heparin in a dose of 400 I.U./kg.

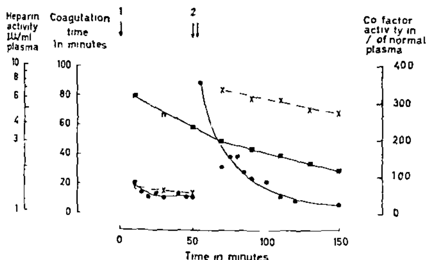


Fig 2 Anticoagulant effect of intravenously injected hepa in with decreased and ncreased heparin co-factor act vity in plasma Heparin dose 400 I U /kg BW 1 Injection of heparin 2 Infusion of fraction IV—I
•— coagulation time
■—■ heparin activity
x—x co-factor activity

Two dogs with normal plasma co-factor activity received a heparin dose of 400 I U /kg followed by repeated doses of fraction IV—1 during the period of elimination of heparin from the plasma

In these experiments the coagulation time was measured every 5 to 10 min using the test tube method previously described

Another two dogs with normal plasma co-factor activity were given a comparatively small dose of heparin *i e* 100 I U /kg of body weight after which the coagulation time was measured every 10 min in a chamber according to JORPES *et al* (1954) One of these dogs was given the heparin first and fraction IV—1 20 min later, in the other dog they were given in the reverse order

In all the experiments listed above the co factor activity in the plasma was measured by the one stage method in samples taken at intervals ranging from 10 to 60 min The heparin level in plasma was also determined

In the 6 experiments on dogs with an *initially decreased plasma co-factor activity* it was 57 75 70 45 78 and 22 % of that in standard plasma respectively In the 4 dogs initially injected with heparin the coagulation time was prolonged to 22 5 22 5 15 and 30 min respectively at heparin levels of about 9 6 5 and 6 I U /ml plasma but then decreased rapidly to pre heparin values despite a high plasma heparin level The co factor activity was however raised and the coagulation time once more prolonged by infusion of fraction IV—I In one of these dogs in which the plasma co-factor activity rose from 60 %

test system. Thus, with no heparin in the test mixture, the "progressive antithrombin" in plasma was measured. At low heparin concentrations fibrinogen exerted a rapid antithrombin effect. At higher heparin concentrations, on the other hand — i.e. between about 2 and 10 I.U./ml of test mixture — another rapid thrombin neutralizing mechanism must be envisaged. The factor in plasma and serum responsible for the lastmentioned effect was tentatively denoted as the heparin co-factor. In the present investigation the heparin co-factor activity has been measured according to this definition.

In the one-stage method described here the antithrombin can be denoted as an "immediately" acting antithrombin. As also in the two-stage method a rapidly acting antithrombin is measured, it is reasonable to suggest that the activity measured by the two methods is identical. The results we obtained were in fact qualitatively similar, even if quantitative discrepancies were observed. The latter might be partly explained by variations in the strength of the thrombin used in the two-stage method. Since in the one-stage method the antithrombin effect is expressed as a percentage of the effect in standard plasma, it is not dependent to the same degree on exact knowledge of the thrombin strength.

The co-factor activity in plasma decreased significantly in both dogs and humans during elimination of the injected heparin from the plasma. MONKHOUSE (1959) observed that the antithrombin titre in plasma decreased *in vitro* after addition of heparin in rising concentrations, as well as after heparin injection in experimental animal. The effect was attributed to an "inhibiting" effect of heparin on antithrombin activity. The results of his *in vitro* experiments are in fact in accordance with those obtained in our previous methodological study (BLOMBÄCK *et al.* 1963). We found that antithrombin mechanisms acting in the absence of heparin and at low heparin concentrations became blocked at higher heparin concentrations, when thrombin inhibition was taken over by the co-factor. The total antithrombin effect of plasma decreased with an increase in heparin concentration. The co-factor activity, however, remained unchanged. Since the latter reaction proceeds stoichiometrically, whereas the reactions at low heparin concentration are strongly dependent on the thrombin concentration, a relatively small proportion of thrombin will be neutralized by the co-factor when thrombin is added in excess as was in fact done by MONKHOUSE. Thus, at excess of thrombin, the total antithrombin effect of plasma will, under certain conditions, decrease with an increase in the heparin concentration. The decrease in antithrombin activity observed by MONKHOUSE following heparin administration *in vivo* is, however, more difficult to ascribe to the heparin co-factor as defined in the present study, since the antithrombin was titrated in the absence of heparin.

REINERT and WINTERSTEIN (1939) suggested that there might be a release of thrombin in the blood secondary to heparin injections. In our test system such a phenomenon should result in higher thrombin activity than that calcu

lated which implies that the decrease recorded in co factor activity would have been only an apparent one However when the co-factor activity was measured at the lowest level in the dogs given a heparin dose of 100 I U /kg of body weight it can be calculated that a plasma thrombin content of 15 NIH units/ml would be required to produce this apparent decrease in anti thrombin activity (see Table I) It would be interesting if such an amount thrombin was present in the circulating blood

The decrease in co factor activity was already measurable 30 min after a heparin injection and seemed to proceed as long as there was any heparin to eliminate However once the heparin was eliminated the co factor activity started to rise again thus indicating the ability of the organism to synthesize co factor A relation obviously exists between the disappearance of heparin from plasma and the decrease in co factor activity The mechanism of co factor decrease is however, difficult to evaluate One possibility is that heparin forms a firm complex with the co factor the complex subsequently being eliminated in some way or another It must however be noted that the curve of decrease in co factor is approximately linear and thus differs from that of heparin which is eliminated exponentially (OLSSON LAGERGREN and [illegible]) Another possible explanation of the co factor decrease is that heparin [illegible] reaction by which an active antithrombin (co factor) is formed from a pro[illegible] which is consumed and that the co factor is continuously eliminated [illegible] blood

It has been demonstrated earlier that the co factor activity [illegible] in Cohn s fraction IV—1 (KOLLER and DEROUAUX 1953, [illegible] anticoagulant effect of heparin at different co factor level[illegible] conveniently be studied by infusing fraction IV—1 [illegible] activity in the plasma produced by infusion of fraction [illegible] accompanied by an increase in coagulation time pro[illegible] contained measurable amounts of heparin The fact [illegible] fraction IV—1 had no effect argues in favour of the [illegible] of native fraction IV—1 is related to the rise in co[illegible] as measured in this test system

The co factor activity in plasma seems to be [illegible] coagulant effect of intravenously injected he[illegible] tionship between heparin and its co factor [illegible] (1943) and VOLKERT (1943) were able to demo[illegible] that both components could be the limiting [illegible] effect of the heparin co-factor complex [illegible] *in vivo* experiments as well In the dogs w[illegible] in plasma the co-factor was the limitin[illegible] seemed to be the limiting component [illegible] coagulation time on repeated infusion [illegible] concentration of heparin in the plasma [illegible]

The present study shows that the prolongation of coagulation time obtained immediately after a heparin injection or a co-factor injection in a heparinized animal is dependent on the level of both heparin and co-factor. It is probable that a certain critical relation must be present between the two components for the coagulation inhibiting effect to be optimal. The semi-quantitative nature of this study does not however permit any relevant calculations. Nor does it explain the rapid decrease in coagulation time despite a high level of both heparin and co-factor activity in the plasma.

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Comparative Effects of Hydralazine, Sodium Nitrite and Acetylcholine on Resistance and Capacitance Blood Vessels and Capillary Filtration in Skeletal Muscle in the Cat

By

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Abstract

ÅBLAD B and S MELLANDER. *Comparative effects of hydralazine sodium nitrite and acetylcholine on resistance and capacitance blood vessels and capillary filtration in skeletal muscle in the cat* Acta physiol scand 1963 *58* 319—329 — The actions of intra arterially infused hydralazine sodium nitrite and acetylcholine were studied with a technique permitting simultaneous and quantitative recording of the responses in the various series coupled sections in the skeletal muscle vascular bed The dilator effects of all three drugs were found to be due to a direct action on the vascular smooth muscle Each of the drugs evoked characteristic peripheral vascular response pattern differentiated from that of the others Thus hydralazine dilated the resistance vessels almost exclusively while sodium nitrite predominantly dilated the capacitance vessels Acetylcholine elicited a pronounced dilatation of both the resistance and the capacitance vessels Furthermore hydralazine and acetylcholine produced a net outward capillary filtration into the extravascular space depending upon a relatively more pronounced dilatation of precapillary than post capillary resistance vessels while sodium nitrite did not significantly influence the transcapillary exchange The present findings may aid in explaining the different general hemodynamic effects elicited by hydralazine and sodium nitrite in the intact organism.

It is well known that the hypotensive effect of hydralazine is due to a decrease of the peripheral vascular resistance. It has been suggested that this effect is caused by an inhibition of the sympathetic vasoconstrictor discharge from the central nervous system (e. g. CRAVER *et al.* 1951). More recent investigations seem however to indicate clearly that the predominant site of action of hydralazine is located in the peripheral vascular bed (STUNKARD, WERTHEIMER and REDISH 1954, ÅBLAD 1959, ÅBLAD, JOHNSSON and HENNING 1961), where the drug produces a decrease of the resistance to blood flow by a direct action on the vascular smooth muscle (ÅBLAD, JOHNSSON and HENNING 1962).

In the intact organism the general hemodynamic effects of hydralazine are partly different from those of nitrites which are also known to exert a vasodilator effect by a direct action on vascular smooth muscle. Thus hydralazine evokes a pronounced decrease in peripheral resistance and an increase of cardiac output (increased heart rate and stroke volume) (e. g. WILKINSON, BACKMAN and HECHT 1952). The nitrites, on the other hand, produce only a slight decrease in peripheral resistance and usually a decrease of cardiac output (based on a decreased stroke volume) (WEISS and ELLIS 1933, BRACHFELD, BOZER and GORLIN 1959, ROWE *et al.* 1961). At present there is no generally accepted explanation for these differences.

In the present study an attempt was made to analyse in some detail the peripheral vascular response patterns induced by hydralazine and sodium nitrite. Such information may be of importance for a more complete understanding of the general hemodynamic effects elicited by the two drugs.

To get a closer insight into the reactions within the peripheral circulatory system it seems important to visualize the functionally differentiated series coupled sections within each vascular bed, e. g. Windkessel vessels, precapillary and postcapillary resistance vessels and capacitance vessels (for details see MELLANDER 1960).

In this study a technique was utilized which permitted simultaneous and quantitative recordings of the abovementioned vascular functions in a skeletal muscle preparation in the hind part of the cat. The effects on the resistance vessels were followed by measuring venous outflow of blood from the region when the pressure gradient across the vascular bed was kept approximately constant. The effects on the capacitance vessels were measured as changes in regional blood content recorded by a radioactive isotope technique. The effects on net transcapillary filtration were deduced from the difference between the recorded changes in regional blood volume and the changes in total volume of the region studied which were measured simultaneously by a plethysmographic technique.

The effects of intra-arterially infused hydralazine, sodium nitrite and acetylcholine were compared to each other. Acetylcholine could be used as a reference agent, since its effects on these vascular functions in great part had been established previously (MELLANDER 1960).

Methods

The experiments were performed on 11 cats weighing 2 8—3 7 kg anesthetized intravenously with a mixture of chloralose (not more than 50 mg per kg) and urethane (not more than 100 mg per kg) In two experiments artificial respiration was given at a level sufficient to just barely suppress spontaneous breathing

The basic experimental technique has been described in detail in a previous communication (MELLANDER 1960) Briefly the hind part of the cat was isolated from its upper part at the level of the hips leaving intact only the abdominal aorta the inferi caval vein and the lumbar sympathetic chains Part of the intestines were removed To obtain an almost pure skeletal muscle preparation the circulation through the hind paws and the tail was excluded by tight ligatures around the ankles and the base of the tail In five of the animals the region studied was deprived of its sympathetic nerve supply by severing the sympathetic trunks at the fourth lumbar ganglia In two animals adrenal medullary secretion was prevented by ligation of both adrenals In these experiments 20 mg cortisone acetate was given i m

In this preparation the inferior vena cava forms the sole outflow channel from the region studied For measuring venous outflow a Gaddum recorder was inserted in the inferior caval vein For calibration the return of blood from the Gaddum recorder was temporarily diverted into a graduated cylinder

The arterial blood pressure was measured by means of a mercury manometer connected to the right carotid artery The venous outflow pressure of the hind part could be set at any desired level by altering the height of the Gaddum recorder abo e heart level

To record the changes in the total volume of the hind part it was enclosed in a water filled temperature regulated plethysmograph connected to a piston recorder By this means rapid phasic changes both in regional blood volume and in regional extravascular fluid volume could be determined as previously described (MELLANDER 1960) To be able to separate exactly these two phenomena also when the vascular reactions developed more slowly simultaneous measurements were made of the changes in regional blood volume by an isotope monitoring technique For this purpose the red cells in the blood were labelled with Cr^{51} The radiation from the region under study was recorded by an external scintillation detector Changes in the recorded activity were a measure of changes in regional blood volume

The scintillation detector (Ekco England) was fitted in a wide angle lead collimator The sodium iodide crystal (diameter 1 5 depth 1) was placed at the side of the hind part and at a distance of 40 cm To record exclusively the radiation from the region enclosed in the plethysmograph all other parts of the animal were shielded by a 3 cm thick lead plate The scintillation detector was connected via an amplifier and a pulse height selector to a linear ratemeter (Ekco England) which operated a chart recorder (Speedomax Leeds & Northrup Co USA) The pulse height selector was set to reject all of the radiation outside the chromium photopeak. The time constant of the ratemeter circuit was set at 1 or 4 sec dependent upon whether rapid or gradual blood volume changes were to be recorded

For labelling approximately 8 ml of blood was withdrawn from the cat early in the preparation and substituted immediately by an equal amount of dextrane Tyrode solution About 2 mC isotonic $Na_2Cr^{51}O$ was mixed with the red cells The labelling procedure was performed according to VEALL and VETTER (1958) The suspension of the labelled red cells was slowly injected i v into the animal Within 10—20 min the recorded radioactivity reached a steady state level varying in different experiments from 250 to 300 cps (background activity about 2 cps) This basal radiation from the region was maintained at approximately the same level throughout the exper

Analysis of blood samples withdrawn during the course of the experiment showed

virtually no radioactivity in the plasma and no hemolysis indicating that the isotope was fixed to the red blood cells.

Changes of the blood volume in the hind part of the cat should be reflected by alterations of the recorded radiation from the region. For a quantitative evaluation of the method a series of experiments was performed in which the regional blood volume was suddenly increased or decreased by a known amount. This was done e. g. by a sudden occlusion of the venous outflow from the region followed within a few seconds by occlusion of the arterial inflow to the region. The increase of regional blood volume was then maintained for about one minute permitting an exact evaluation of the change in radiation. A similar steady state change in regional blood volume could also be produced by a sudden graded partial occlusion of the venous outflow from the region, by an infusion of acetylcholine or by electrical stimulation of the sympathetic vasoconstrictor fibres to the region. It has previously been shown that the changes in regional blood volume produced by these manoeuvres can be measured quantitatively with a high degree of accuracy by the plethysmographic volume recording (MELLANDER 1960). The concomitantly recorded changes in regional blood volume by the isotope monitoring technique showed a satisfactory correlation to the values obtained by the plethysmographic technique, the standard deviation in 68 such determinations amounting to ± 11 %.

For routine calibration of the isotope blood volume technique, made repetitively during the course of each experiment, the method of graded partial occlusion of the venous outflow was utilized.

The drugs were administered i. a. to the region from a constant rate infusion apparatus connected to an indwelling catheter in the inferior mesenteric artery. The infused amounts of acetylcholine ranged between 0.01 and 0. μg/min/kg of studied region, hydralazine (Apresolin, Ciba) between 0.12 and 0.16 mg/min/kg, sodium nitrite between 0.1 and 1.0 mg/min/kg. Acetylcholine was infused for periods of 1—3 min, hydralazine and sodium nitrite for 4—8 min. The interval between the infusions of hydralazine and sodium nitrite was 40 min as a minimum. In about half of the experiments hydralazine was given before sodium nitrite, in the remainder the sequence was reversed. Acetylcholine was given repetitively in all experiments both before and after each of the above mentioned drugs.

With the present technique, phasic changes in blood flow, blood volume and in net transcapillary filtration exchange induced by acetylcholine, hydralazine and sodium nitrite could be recorded continuously while at the same time the arterio-venous pressure gradient across the vascular bed could be kept fairly constant.

Results

Fig. 1 shows the original recordings from a representative experiment in which acetylcholine (A), hydralazine (B) and sodium nitrite (C) were infused i. a. to the skeletal muscle region in the hind part of the cat during the periods indicated by the signal. The lumbar sympathetic nerve trunks were cut in this experiment. In the amounts given neither of the drugs produced a significant change of the arterial blood pressure. It can be seen that all drugs produced an increase of the blood flow and an increase of the volume of the region studied. The responses of the resistance vessels were deduced by calculating the regional resistance in peripheral resistance units (GREEN 1948 p. 243) immediately before each infusion and at the point at which a steady state effect in blood flow was obtained. The extent of dilatation of the resistance vessels for each drug is indicated in the

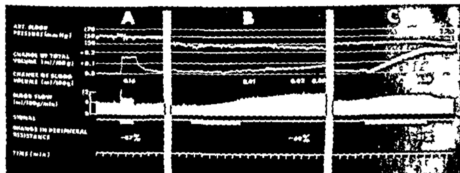


Fig 1 Cat 2.8 kg chloralose urethane sympathectomized skeletal muscle region 460 g The effects on resistance and capacitance vessels and on net transcapillary exchange evoked by intra arterial infusion of A) acetylcholine (0.10 μg/min/kg of studied region) B) hydralazine (0.12 mg/min/kg) and C) sodium nitrite (0.6 mg/min/kg) For details see text

figure by the percentage decrease in regional peripheral resistance The responses of the capacitance vessels and the net transcapillary filtration exchange were obtained from the plethysmographic recording of the change in total volume and from the simultaneously recorded change in regional blood volume by the isotope monitoring technique As has been established previously (MELLANDER 1960) the plethysmographic volume recording alone can give information about both these phenomena provided the vascular responses develop rapidly as for example with acetylcholine (A) Then the initial rapid increase in volume represents the amount of blood pooled in the region, and indicates the response of the capacitance vessels The latter more slowly changing portion of the volume curve reflects changes in net capillary filtration When the vascular responses develop more gradually as occurred when hydralazine and sodium nitrite were administered such an exact differentiation between these two phenomena can no longer be made on the basis of the plethysmographic volume curve alone but required in addition simultaneous measurements of the changes in regional blood volume as accomplished by the isotope technique Therefore with this method the responses of the capacitance vessels could be assessed more exactly from the recorded changes in regional blood volume These values are indicated in the figure by the numbers below the volume curve Consequently a net transcapillary outward filtration could be determined by subtracting the value for the recorded blood volume increase from the value for the recorded increase in total volume

As can be seen in Fig 1 all three drugs dilated both the resistance and the capacitance vessels and for each substance these two vascular functions seemed to be fairly well coordinated in time to each other

Acetylcholine (A) produced an almost immediate dilator response of both the resistance and capacitance vessels reaching a steady state level within 20—30 sec The effect was completely abolished 1—2 min after the cessation of the

infusion. In contradistinction to this the dilator responses of hydralazine (B) were characterized by a much more gradual development reaching a steady state level about 12—14 min after the start of the infusion or only several minutes after the infusion ceased. These dilator effects were then maintained for considerable length of time and had sometimes not disappeared even one or two hours later. Sodium nitrite (C) finally, had in this respect an intermediate position as compared to acetylcholine and hydralazine in that the dilator responses of the resistance and capacitance vessels showed a fairly rapid onset reaching a steady state level about 5—7 min after the start of the infusion. The effect was maintained for 15—30 min after the end of the infusion. The results from some experiments suggested that with sodium nitrite the recovery of the resistance vessels was somewhat more rapid than that of the capacitance vessels.

A comparison between the effects of the three drugs on the resistance and capacitance vessels revealed marked differences from a quantitative point of view. Acetylcholine (A) evoked a relatively great dilatation of both the resistance and capacitance vessels. Hydralazine (B) on the other hand elicited a pronounced dilatation of the resistance vessels while concomitantly the dilator response of the capacitance vessels was extremely small. Sodium nitrite (C) finally produced only a moderate dilatation of the resistance vessels but a far more pronounced dilatation of the capacitance vessels than hydralazine. The experiment shown in Fig. 1 further demonstrated that the three drugs influenced the capillary filtration transfer to a different extent. As mentioned above the magnitude by which the increase in total volume (plethysmographic method) exceeds the increase in regional blood volume (isotope method) is a measure of transcapillary outward filtration. For sodium nitrite (C) there was a close correlation between the recorded change of total volume and blood volume in the region which indicated that no significant change in net filtration transfer occurred. Acetylcholine (A) and hydralazine (B) on the other hand produced a greater increase of total volume than of blood volume thus demonstrating an outward filtration process. For acetylcholine this slight but consistent difference could be more easily seen if the infusion period was further prolonged. A quantitative estimation of this phenomenon is most readily obtained at the stage at which the resistance and capacitance vessels have reached a steady state dilatation. The slow continuous upward inflection of the plethysmographic volume curve at this stage is then a measure of the rate of outward filtration. The filtration rate so determined was in this particular experiment approximately twice as great for hydralazine as for acetylcholine. This fluid transfer into the extravascular space is related to a decrease in the ratio of precapillary to postcapillary resistance as will be discussed below.

It was further found that the amplitude of the oscillations synchronous with the pulse wave seen in the plethysmographic recording were considerably augmented after infusion of sodium nitrite (Fig. 1 C) but only slightly changed

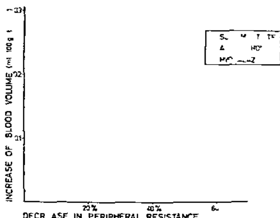


Fig 2 Diagram showing the relation between the dilator effects on the resistance vessels (decrease in regional resistance) and capacitance vessels (increase in regional blood volume) produced by i a. infusions of hydralazine acetylcholine and sodium nitrite Each point represents a single observation of the effects obtained during steady state condition Note that hydralazine predominantly affected the resistance vessels and sodium nitrite predominantly the capacitance vessels.

with the two other drugs Owing to the slow paper speed each oscillation cannot be distinguished here but the increased amplitude is clearly demonstrated in the pen recording (C) This seems to indicate that sodium nitrite relaxed the smooth muscles of the Windkessel vessels which would tend to increase their distensibility This would furthermore imply a slight increase of the blood volume within the Windkessel vessels which would contribute to the regional capacitance response

The above described general patterns of vascular response for the three drugs including the different reactions of the resistance capacitance and Windkessel vessels and the effects on the capillary filtration transfer were observed in all the present experiments Thus these patterns were found both in experiments where the sympathetic nerve fibres were left intact and when the region was deprived of its extrinsic sympathetic control and furthermore regardless of an intact or eliminated adrenal medullary secretion Neither was any major change from this general pattern noted when as occurred in a few experiments the systemic arterial blood pressure decreased slightly during the infusions The above described observations indicate that the vascular responses elicited were predominantly due to a peripheral site of action of the drugs in the region studied

One of the more outstanding features of the present study seems to be the observation that hydralazine and sodium nitrite evoked so different responses from a quantitative point of view in the resistance and the capacitance vessels Fig 2 shows the dilator effects obtained at steady state in the resistance and the capacitance vessels for all three drugs investigated and includes the results from all experiments The data are here plotted in terms of the induced increase in regional blood volume *versus* the induced decrease in regional resistance representing the effects on the capacitance vessels and resistance vessels respectively It can be seen that for each drug the values are grouped in a

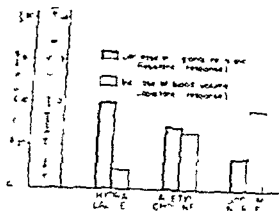


Fig. 3. Mean values for decrease in regional resistance and increase in regional blood volume evoked by hydralazine (13 exp.), acetylcholine (3 exp.) and sodium nitrite (7 exp.)

uniform manner with the relatively greatest effect on the capacitance vessels exerted by sodium nitrite and the relatively greatest effect on the resistance vessels exerted by hydralazine. For acetylcholine the values are grouped in the intermediate zone. This general tendency may be more clearly shown in Fig. 3 where the mean values for all data are indicated. It should be noted that the diagram of Fig. 2 includes values obtained both when hydralazine was given before sodium nitrite and when the sequence was reversed. The values for acetylcholine represents data obtained by infusion of the drug both before and after administration of the two other substances. It can therefore be concluded that the general pattern of response was not dependent upon the sequence of administration of the drugs.

Discussion

In recent studies (ÅBLAD *et al.* 1961, ÅBLAD and JOHNSSON 1963) it was demonstrated that intra-arterially administered hydralazine and sodium nitrite to the human forearm produced a decrease in vascular resistance which from a quantitative point of view was similar to the effects obtained in this study on cat skeletal muscle. Furthermore, the effects on man and in the cat seemed to be well coordinated in time to each other. Such observations may suggest that the peripheral vascular response patterns for the two drugs are similar in these two species.

The results of the present study indicate that the peripheral vascular response of the infused hydralazine was very similar in the innervated and sympathectomized skeletal muscle region, which confirms other studies (STUNKARD, WERTHEIMER and REDISCH 1954, ÅBLAD *et al.* 1962) showing that its dilator effect is due predominantly to a direct action on the vascular smooth muscle and not primarily to a peripheral inhibition of the sympathetic vasoconstrictor tone. Similarly, as expected, the effects of acetylcholine as well as of sodium nitrite, agents known to have a direct peripheral action, were comparable

from a quantitative point of view in the innervated and sympathectomized preparation

The present study indicates that hydralazine predominantly dilated the resistance vessels situated mainly on the precapillary side of the circulation (i e small arteries and arterioles) while sodium nitrite predominantly dilated the capacitance vessels situated mainly on the postcapillary side of the vascular bed (i e venules and veins) When the amounts of the two drugs were so adjusted as to produce an equal dilatation of the resistance vessels sodium nitrite evoked a capacitance response several times larger than that of hydralazine Furthermore under such circumstances sodium nitrite seemed to produce a much more pronounced relaxation of the smooth muscles of the Windkessel vessels than hydralazine From this it can be concluded that the two drugs evoked distinctly different peripheral vascular response patterns with regard to the various series coupled sections within the same vascular bed Theoretically such a difference in the mode of action of the two drugs may be explained in several ways At present the most reasonable explanation seems to be that the smooth muscle cells of the different series coupled vascular sections are characterized by different degree of sensitivity to hydralazine and sodium nitrite

As has been demonstrated previously (MELLANDER 1960) and as was shown in the present experiments acetylcholine produced beside the effects on the resistance and capacitance vessels also a net outward movement of intravascular fluid The mechanism behind this phenomenon has been discussed in detail in the previous communication and will here be mentioned only briefly Under the prevailing experimental conditions of this study a change in capillary fluid filtration transfer can be explained only by a change in mean hydrostatic capillary pressure This in turn must be dependent upon a change in the relation between pre and postcapillary resistances Acetylcholine thus produced a relatively greater dilatation of the precapillary resistance vessels than of the postcapillary resistance vessels or in other words decreased the pre to postcapillary resistance ratio in turn leading to a rise of mean hydrostatic capillary pressure which explains the net outward filtration

In comparison to acetylcholine hydralazine often seemed to produce an even greater decrease of the pre to postcapillary resistance ratio to judge from the greater rate of outward filtration observed in the present experiments Sodium nitrite on the other hand seemed to hardly affect the pre to postcapillary resistance ratio since its effect on the net filtration transfer was found to be insignificant This interpretation may be further supported by the fact that hydralazine predominantly dilated the resistance vessels (the most important ones located to the precapillary side) while sodium nitrite beside a slight effect on the resistance vessels evoked a pronounced dilatation of the capacitance vessels (mainly postcapillary and in all probability including also the lary resistance vessels)

In a recent study (ÅBLAD and JOHNSSON 1963) the effects of i. a. infused hydralazine and sodium nitrite on the vascular resistance and the total volume of the human forearm were investigated. In comparison to sodium nitrite, hydralazine was found to produce a relatively greater decrease of the vascular resistance but a relatively smaller increase of the total volume of the forearm. *With the technique used it was*, however, not possible to make a differentiation between changes in regional blood volume and changes in extravascular fluid volume, which both would contribute to the induced changes in total forearm volume. The results of the present study are in agreement with those obtained in man in that hydralazine produced a relatively greater decrease of vascular resistance and a relatively smaller increase of total volume than sodium nitrite. Therefore, it seems likely that the above described differentiated vascular response patterns in cat skeletal muscle regarding precapillary and postcapillary resistance vessels, capacitance vessels and capillary filtration transfer will also be found in the human forearm during hydralazine and sodium nitrite administration.

Provided the vascular reactions above described would be the dominating ones as regards the general vascular response pattern in the systemic circulation one would expect that hydralazine should decrease the total blood volume but hardly change peripheral vascular blood capacity while sodium nitrite should not significantly alter the total blood volume but considerably increase peripheral vascular blood capacity. However, one would also expect that in the intact organism the induced changes in arterial and venous pressures directly and indirectly by nervous reflex adjustments might to some extent modify such changes in the blood volume and its distribution. *In a recent study in man* (ÅBLAD 1963) in which changes of total blood volume and pulmonary blood volume were determined it was found that i. v. administered sodium nitrite produced some increase in total blood volume but simultaneously a relatively much more pronounced increase of peripheral vascular blood capacity. An equihypotensive dose of i. v. administered hydralazine produced a slight decrease of the total blood volume but simultaneously an equivalent decrease in peripheral vascular blood capacity, probably caused by a reflex constriction of the capacitance vessels.

As mentioned in the introduction the hypotensive effect of nitrites is due to a great extent to a decreased cardiac stroke volume whereas the peripheral vascular resistance is but slightly decreased. It has previously been suggested on the basis of more or less indirect evidence that the reduced stroke volume is due to a decreased venous return, depending upon an increased peripheral vascular blood capacity. This study, in which the peripheral vascular response pattern was investigated by a more direct approach seems to provide further evidence in support of this hypothesis.

The present finding that hydralazine is a potent dilator of resistance but not of capacitance vessels may help to explain why this drug in the intact organism

produces a pronounced decrease of peripheral resistance but an increased stroke volume *This latter effect may be a consequence of the effects of increased* activity in the sympathetic nerves to the heart (see MOYER HANDLEY and HUGGINS 1953) in the face of no change or even a decrease in peripheral vascular blood capacity

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Studies on the Excretion Mechanism of Serotonin (5-Hydroxytryptamine) in the Chicken Kidney

By

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Abstract

SANNER, E. *Studies on the excretion mechanism of serotonin (5-hydroxytryptamine) in the chicken kidney.* Acta physiol. scand. 1963. *58.* 330–341. — Serotonin at a dose of 150–200 µg was injected into the leg vein of the chicken together with phenol red and tolazolin. All birds were pre-treated with a MAO inhibitor (JB-516, Catron). *The tubular excretion of serotonin was inhibited by simultaneous administration of* tolazoline at a dose of [illegible]–15 mg into the leg vein. Probenecid at a dose of [illegible] mg/kg given into the wing vein 1 hour prior to serotonin — phenol red injections gave a strong inhibition of phenol red excretion but left the serotonin excretion unchanged. Bromcresol green at a dose of 1 mg caused a specific inhibition of phenol red transport but left serotonin transport unchanged. Cyanine dye # 863 at a dose of *2 mg did inhibit the serotonin transport but such a big dose also interfered* with phenol red transport capacity. Smaller doses of cyanine dye gave a weak inhibition of serotonin transport and did not alter the phenol red transport to any great extent. Reserpine did not inhibit either serotonin or phenol red transport. pH of the chicken urine *did not influence the excretion of serotonin.*

It has been recently shown in the chicken that serotonin (5-hydroxytryptamine) *is excreted by an active tubular excretion mechanism* (SANNER and WORTMAN 1962). In order to study the nature of this excretion mechanism the following investigation has been done. The transport mechanism for serotonin can obviously be the same as that for many bases. *It was very improbable that the transport* mechanism for acids (phenol red system) could also be involved but for completeness this was also tested. In an earlier work (SANNER and WORTMAN 1962) it is mentioned that the transport mechanism *for serotonin*

might possibly be inhibited by reserpine and thus there could possibly be one transport mechanism common to many bases and one transport mechanism sensitive to reserpine inhibition In order to settle this question some of the experiments were performed on reserpine pre treated birds The influence of the pH of the chicken urine on the excretion of serotonin was also tested

Material and methods

The general procedure was essentially the same as earlier described (SANNER and WORTMAN 1962) Campbell s modification of the original Sperber technique was used (CAMPBELL 1960 SPERBER 1949)

A Animals Rhode Island Red chickens about two years old weighing 2 0—3 5 kg were used They were kept on commercial chicken food and fresh water ad libitum Generally the birds were kept fasting over night before the experiment The birds were unanesthetized and kept in sitting position during the experiment Small plastic funnels were sutured over each ureteral orifice under topical anesthesia (5 % Lidocain ointment) The funnels were irrigated with a constant flow of distilled water at a rate of 0 5 ml/min on each side to prevent clogging of the funnels by uric acid The urine was collected from each side separately According to previous experience only birds pre treated with a mono amine oxidase (MAO) inhibitor can be used in such a study because of the fast breakdown of serotonin by the MAO in the chicken kidney The substance JB 516 (1 phenyl 2 hydrazinopropane Catron® Lakeside) was therefore always given into the wing vein at a dose of 10 mg/kg at least 1—2 hours before the start of the injections That this dose inhibits MAO to 100 per cent in the rat has been shown by HORITA and MCGRATH (1960)

B Solutions

a Serotonin Serotonin creatinine sulphate (Sandoz) was provided in ampoules containing 10 mg/ml of the complex All serotonin solutions were prepared on the day of the experiment by diluting with redistilled water to 150—200 μg/ml (pH = 7) The serotonin solution was shown to be stable at room temperature for at least 18 hours At 26 hours the serotonin activity had fallen somewhat

b Tolazoline Tolazoline (Vasodil® Leo Halsingborg) was dissolved in 0 9 per cent saline to contain 1 000 μg/ml (pH = 5 6) 5 000 μg/ml (pH = 5 4) or 15 000 μg/ml (pH = 5 2) respectively on the day of the experiment

c Cyanine dye The cyanine dye # 863 (1 ethyl 3 6 -dimethyl 2 phenyl-4 pyrimido-2 cyanine) was dissolved in 0 9 per cent saline to 1 000 μg/ml or 2 000 μg/ml on the day of the experiment

d Phenol red and brom cresol green Both the solutions were prepared in 0 9 per cent saline to contain 1 000 μg/ml (pH 7)

e Reserpine Serpasil® (Ciba Basel) was used from ampoules containing 2 5 mg/ml (pH = 4)

C Injection technique

Leg vein injections Generally 400 μg or 800 μg of phenol red (0 4 or 0 8 ml respectively) was injected into one leg vein of the chicken at the beginning of the experiment Those very few chickens that did not show a visible excess excretion of phenol red on the injected side were discarded The injections of serotonin were made into the leg vein together with phenol red and tolazoline (at most 2 8 ml) All solutions were mixed

the same system except in a few experiments (no. 438, 442, 451) where the basic cyanine dye ([illegible]) was injected into the leg vein just before the other test solutions. In some experiments (no. 410, 411 and 419) reserpine was also injected into the leg vein just before the other solutions.

The leg vein injections usually lasted for 3 min and were followed by a urine collection period of 3 × 20 min. At least 70 min elapsed between every injection. Generally 3 to 4 injections were made on the same hen.

Pretreatment. In 2 experiments (no. 437, 440) 10 mg/kg or 50 mg/kg tolazoline respectively was injected in one wing vein *1 hour before the main experiment.* In 2 experiments (no. 433, 441) probenecid 50 mg/kg was injected into the wing vein 1 hour before the main experiment. *In 2 experiments (no. 452, 464) the chicken was given* [illegible] sodium bicarbonate into the wing vein in order to produce a neutral or slightly alkaline urine. The birds were primed with 100 *mg/kg sodium bicarbonate. Thereafter* about 10 mg/kg/min was infused throughout the experiment. In order to achieve a steady state 30 min was allowed before the *leg vein injections. In one of these birds also* 3 mg/kg acetazolamide was given systemically before the serotonin phenol red injection.

Pretreatment with reserpine. Reserpine 1 mg/kg was *injected in the pectoral muscle* 17—19 hours before the main experiment in 2 experiments (no. 447, 453).

C. Collection of urine. The diluted chicken urine was collected at 20 *min periods in* test tubes containing 1.0 ml 0.4 N perchloric acid in order to stabilize the serotonin. In experiments which included pH measurements no perchloric acid was added to the tubes. The addition of perchloric acid was also later shown to be unnecessary by the following experiment.

Two serotonin solutions of 4 *μg*/ml were prepared in diluted chicken urine and kept in the refrigerator at + 4° C. In one of them 1.0 ml of 0.4 N perchloric acid *per 10 ml* diluted chicken urine was added. These two solutions were analysed for their serotonin content and were shown to be stable for 12 days and then the serotonin activity fell about equally in both the solutions.

D. *Analyses*

a. Serotonin. The analyses for serotonin were made with a *modification of the extraction* methods described by UDENFRIEND, WEISSBACH and CLARK (1955).

1) *2 ml* of diluted chicken urine with 0.4 N perchloric acid *(in the approximate* proportions of 13:1) was added to 4.0 ml 0.1 N hydrochloric acid.

2) pH was adjusted to pH 10 with a small *amount of water free sodium* carbonate.

3) 5 ml borate buffer (pH 10) was added and redistilled water was added up to 15 ml volume.

4) 5 g sodium chloride and 15 ml butanol was added, then shaken for 10 min and centrifuged *at low speed for 5 min.*

5) The water phase was pipetted away.

6) *The remaining butanol* phase was added to an equal amount of borate buffer, shaken for 10 min and centrifuged for 5 min.

7) *10 ml of the butanol phase* was transferred into a flask containing 20 ml heptane and 1.5 ml 0.1 N hydrochloric acid. *It was shaken* for 10 min and centrifuged for 5 min.

8) *One ml of the acid water* phase was transferred into a cuvette containing 0.3 ml of conc. hydrochloric acid. The solutions were activated at 300 m*μ* and the fluorescence was read in an Aminco-Bowman spectrophotofluorometer at [illegible]40 m*μ*.

Recovery experiments were performed by preparing serotonin solutions in diluted chicken urine at *concentrations* from 0.5 *μg*/ml—6.0 *μg*/ml. The average recovery was 106 per cent in 20 determinations (3 at each level except for 0.5 *μg*/ml where 2 determinations only were *performed).*

Table I Tola_oline experiments

A Tolazoline wing vein.

Expt no	Injected dose			ATEF 100		Recovery per cent of dose		Inj time (min)
	5 HT µg, leg vein	Ph. Red µg leg vein	Tolazoline —1 hour wing vein	5 HT	Ph Red	5 HT	Ph. Red	
439	—	800	—	—	42 9	—	77 5	5
	200	800	—	17 8	43 4	17 8	74 ?	6
	200	800	10 mg/kg	55 1	55.3	56 1	84 6	7
440	—	800	—	—	20 0	—	67 8	5
	200	800	—	36.5	13 7	42 6	66 9	8
	200	800	50 mg/kg	3.5	7 7	4 4	67 4	6

B Tolazoline leg vein.

Expt. no.	Injected dose µg leg vein			ATEF 100		Recovery per cent of dose		Inj time (min)
	5 HT	Ph Red	Tolazoline	5 HT	Ph. Red	5 HT	Ph. Red	
433	150	400	—	33 4	17 8	33 4	62.1	8
	150	—	—	39 4	—	39 4	—	8
	150	400	800	39 9	32 8	40 8	68 4	8
	—	400	—	—	30 6	—	73 3	8
441	—	800	—	—	22.1	—	66 3	3
	200	800	—	56.2	19 3	56 8	71 4	3
	200	800	1 500	3? 3	24 3	33 1	71 4	3
	200	800	5 000	12 3	27 9	14 3	70 5	3
442	—	,00	—	—	15 4	—	61 3	3
	200	800	—	33 5	27 7	39 4	61 1	3
	200	800	5 000	6 4	16 6	6 4	51 0	3
	200	800	15 000	4 3	?3 9	4 3	53 7	3
444	—	800	—	—	19 0	—	69 9	3
	200	800	—	34 3	40 4	38 4	50 5	3
	200	800	5 000	7 6	18 0	11 2	60 2	3
	?00	800	15 000	7 8	23 0	7 8	63 5	3
Mean (no of determ)								
(5)	—	800	—	—	23 9	—	68 6	
(5)	200	800	—	35 7	28 9	39.2	64 8	
(1)	200	800	1,500	3?.3	24 3	35 7	71 4	
(3)	200	800	5 000	8.8	.0 8	10 6	60 6	
(?)	00	800	15 000	6 1	23.5	6 1	58 6	

Table II [illegible]

Exp n	Injected dose µg leg vein 5-HT	Ph. Red	T. lazo line	Pr-treated Wing vein —1 hour	ATEF · 100 5-HT	Ph. Red	Recovery per cent of dose 5-HT	Ph. Red	Inj. time (min)
433	—	800	—	—	—	3 7	—	38	5
	200	800	—	—	47 2	33 5	5 4	64	5
	200	800		50 mg kg	47 1	5.2	4 1	5 5	5
445	—	800	—	—	—	18.1	—	57	3
	200	800	—	—	44.2	14 4	50 5	6 8	3
	200	800	—	50 mg kg	47.2	3 5	47.2	33 7	3
	200	800	15 000		9	−3 8	6 6	39.5	3
Mean (no of determ)									
(?)	—	800	—			27 9		48	
(?)	00	800	—		46 7	4 0	51 5	7 1	
(?)	200	800	—	50 mg kg	4 2	4 4	41 7	29 6	
(1)	00	800	15 000		9	−3 8	6 6	39 5	

b *Phenol red*

The phenol red was measured colorimetrically at an alkaline pH When phenol red was the only dye injected a Zeiss Elko photometer was used at a wave length of 530 mµ (filter S 53E) When brom cresol green was injected together with phenol red the extinction of phenol red was measured in a Beckman B spectrophotometer at 565 mµ (uncorrected apparatus value) in order to be able to distinguish between the two dyes in the sample

The excretion is given as the apparent tubular excretion fraction (ATEF) according to SPERBER (1948) The difference between the amounts excreted by the ipsilateral and the contralateral kidney divided by the dose injected is taken as a measure of the tubular excretion of the injected substance (ATEF)

Results

1 Phenol red given to normal chickens

In normal chickens pre treated with JB 516 phenol red at a dose of 800µg injected into the leg vein gave a mean ATEF of 26 9 ± 2 9 % (12 expts)

2 Serotonin — phenol red injections to normal chickens

In normal chickens pre treated with JB 516 serotonin at a dose of 200 µg given together with 800 µg of phenol red into the leg vein gave a mean ATEF of 33 8 ± 3 2 % for the serotonin and 26 2 ± 2 8 % for the phenol red (14 expts) Thus serotonin did not interfere with the excretion of phenol red in the dose range used

Table III Brom cresol green experiments

Expt. no	Injected dose, µg leg vein			ATEF 100		Recovery per cent of dose		Inj time min)
	5 HT	Ph. Red	Br cr green	5 HT	Ph. Red	5 HT	Ph. Red	
465	200	800	—	20 2	13 5	23 1	61 8	3
	200	800	1 000	20 4	1 3	27 6	54 7	3
	—	800	—	—	26 9	—	83 8	3
	—	800	1 000	—	—1 9	—	55.8	3
467	—	800	—	—	13.2	—	85 0	3
	200	800	—	15 5	7 9	27 4	63 8	3
	200	800	1 000	18 0	—0.5	18 9	72 8	3
	—	800	1 000	—	5 1	—	63 7	3
469	—	800	—	—	37 7	—	83 3	3
	200	800	—	40 0	22 6	45 0	87.2	3
	200	800	1 000	33.2	5 8	39 5	77.2	3
	—	800	1 000	—	5 3	—	77 0	3
Mean (no of determ)								
(3)	—	800	—	—	24 3	—	84 0	3
(3)	200	800	—	25 2	14 7	30.2	71 3	3
(3)	200	800	1 000	23 9	2 2	27 0	66 6	3
(3)	—	800	1 000	—	2 8	—	65 5	3

In experiment no 433 the excretion of serotonin alone was compared to that of a mixture of serotonin and phenol red Here 150 µg of serotonin alone gave an ATEF of 39 5 % and when the same dose of serotonin was given together with 400 µg of phenol red the ATEF was 33 4 % for serotonin and 17 8 % for phenol red Thus phenol red did not have any influence on the tubular excretion of serotonin in the dose range used

3 Tolazoline experiments

a Tolazoline given into the wing vein

Tolazoline at a dose of 10 mg/kg given into the wing vein 1 hour before the experiment did not inhibit the tubular transport of either serotonin or phenol red If anything the tubular excretion of serotonin increased at that dose of tolazoline (expt no 439 Table I A)

When tolazoline was given into the wing vein at a dose of 50 mg/kg 1 hour before the injections both the serotonin and phenol red transport were strongly inhibited Recovery of serotonin was low but that of phenol red normal however (expt no 440 Table I A)

Table II. Cyanine experiments

Expt no.	Injected dose µg leg vein			ATFF (%)		Recovery per cent of dose		Inj. time (min)
	5-HT	Ph. Red	Cyanine	5-HT	Ph. Red	5-HT	Ph. Red	
443	—	800	—	—	[illegible]	—	[illegible]	3
	200	800		3 .5	32.0	34.5	69.9	3
	200	800	1 000	[illegible] 7	32.2	2 7	68.8	3
4 [illegible]	—	800	—	—	41.7	—	[illegible] 6	3
	200	800	—	41.2	3 [illegible]	4 .2	63.9	3
	200	800	1 000	29.8	33.5	30	68.6	3
451	—	800		—	18.4	—	80.3	3
	200	800	—	[illegible] 6	1 1	35.8	0.9	3
	200	800	2 000	17.8	8.4	21.9	69.0	3
Mean (no. of determ.)								
(3)	—	800		—	29.2	—	74.3	3
(3)	200	800		31.4	8.9	3 5	68.2	3
(2)	200	800	1 000	28.3	33.9	29.	68	3
(1)	200	800	2 000	1 8	8.4	21.9	69.0	3

b) *Tolazoline given into the leg vein*

When the tolazoline substance was administered into the leg vein together with serotonin and phenol red the following results were apparent. At a dose of 800 µg (during 8 min) tolazoline did not give any alteration of either serotonin or phenol red excretion (expt. no. 433 Table I B). At a dose of 1 500 µg of tolazoline (injection time 3 min) a small inhibition of serotonin transport was produced (expt. no. 441).

When the tolazoline dose was increased to 5 000 µg, however, there was an almost complete inhibition of the serotonin transport (mean ATFF = 8.8 %) but phenol red excretion was not influenced at all (3 expts.).

In 2 experiments the dose of tolazoline was increased to 15 000 µg and this promoted, if possible, an even greater inhibition of serotonin excretion (mean ATFF = 6.1 %) but also here the phenol red excretion was left unaffected.

4. *Probenecid experiments*

Probenecid at a dose of 50 mg/kg into the wing vein 1 hour before the experiment gave a deep inhibition of phenol red excretion but left the serotonin excretion unchanged (2 expts.). One such a probenecid pre-treated bird was used for an experiment where tolazoline at a dose of 15 000 µg was injected into the leg vein together with phenol red and serotonin. Here the serotonin excretion

Table I a. Reserpine experiments

1 mg kg reserpine was injected i m. pectoralis 17—19 hours before

Expt. no	Injected dose μg leg vein				ATEF 100		Recovery per cent of dose		Inj time (min)
	5-HT	Ph. Red	Tolazoline	Cyanine	5-HT	Ph. Red	5 HT	Ph Red	
447	—	800	—	—	—	18.2	—	68.5	3
	200	800	—	—	29.2	25.3	35.3	74.4	3
	200	800	5 000	—	14.7	34.9	19.6	77.8	3
	200	800	—	500	19.2	31.2	31.4	71.4	3
455	—	800	—	—	—	30.6	—	79.3	3
	200	800	—	—	32.7	17.8	36.5	56.7	3
	200	800	5 000	—	12.5	29.5	15.7	8.2	3
Mean (no of determ)									
(2)	—	800	—	—	—	24.4	—	73.9	3
(2)	200	800	—	—	31.0	21.6	35.9	65.6	3
(2)	200	800	5 000	—	13.6	32.2	17.7	78.0	3
(1)	200	800	—	500	14.7	31.2	31.4	71.4	3

was strongly inhibited and the phenol red excretion remained at a low value (expt no 445 Table II)

5 *Brom cresol green experiments*

Brom cresol green at a dose of 1 000 μg (during 3 min) was injected into the leg vein of the chicken together with serotonin and phenol red Here as in the probenecid experiments the phenol red excretion was strongly inhibited but the serotonin excretion was not changed (3 expts Table III)

6 *Cyanine dye experiments*

The basic cyanine dye # 863 was given into the leg vein just prior to the injections of serotonin phenol red into the same vein In 2 experiments cyanine at a dose of 1 000 μg gave a small inhibition of serotonin transport but did not alter the phenol red transport In one experiment cyanine was given at a dose of 2 000 μg Here the transport of serotonin was somewhat inhibited but the phenol red excretion was also clearly depressed compared to normal controls (Table IV)

7 *Reserpine experiments*

In 2 experiments (no 447 455 Table V A) reserpine 1 mg/kg was given into the pectoral muscle 17—19 hours before the main experiments The mean

Table V B

Reserpine injected into one leg vein.

Expt no.	Injected dose µg leg vein			ATEF 100		Recovery per cent of dose		Inj. time (min)
	5-HT	Ph. Red	Reserpine	5-HT	Ph. Red	5-HT	Ph. Red	
[illegible]	[illegible]00	—	[illegible]00	32.5	—	3[illegible]2	—	3
	—	800	—	—	37.1	—	60.1	3
510	[illegible]	800	—	19.9	[illegible]8.8	[illegible]3.0	9.1	3
	[illegible]00	800	[illegible]00	17.0	[illegible]8	[illegible]7.9	5[illegible]9	3
511	[illegible]00	800	—	25.9	11.6	31.3	5 0	3
	200	800	2,500	47.8	31.1	55.7	[illegible]5.9	3
519	[illegible]00	800	2,[illegible]00	3[illegible]4	11[illegible]	41.1	[illegible]9.0	3
Mean (no. of determ.)								
(1)	—	800	—	—	35.1	—	60.1	3
( )	200	800	—	[illegible]9	[illegible]1.7	[illegible]9.7	[illegible]8.1	3
(1)	[illegible]00	—	2,500	3[illegible]3	—	3[illegible]2	—	3
(2)	[illegible]00	800	2 [illegible]00	[illegible]9.9	28.5	41.8	61.4	3
(1)	[illegible]00	800	2 [illegible]00	3[illegible]4	14.2	41.1	39.0	3

serotonin A I L I in these two reserpine pre treated birds was 31 0 % as against 33 8 ± 3 2 % in 12 non reserpinized chickens Tolazoline at a dose of 5 000 µg was then given together with serotonin and phenol red into the leg vein This procedure gave *excretion values for serotonin which were smaller than normal* controls but the inhibition certainly was not stronger than in nonreserpinized chickens

In one reserpine pre treated bird (expt no 447) the basic cyanine dye # 863 was injected *into the leg vein at a dose of 500 µg together with 200 µg sero-*tonin and 800 µg phenol red This possibly gave a small inhibition of serotonin excretion The phenol red excretion was in the same range as in the previous injection with tolazoline in the same bird (Table V A)

In 2 experiments (*no 510 511 Table V B) 2 5 mg reserpine* was *injected* into one leg vein just before the serotonin and phenol red injections into the same leg vein This injection procedure was considered necessary because of a tendency of reserpine to precipitate in the syringe together with serotonin and phenol red In one earlier experiment (no 506) where *no such precipitation could be observed* 2 5 mg reserpine was injected into one leg vein together with 200 µg serotonin The results did not show any inhibition of serotonin phenol red transport by reserpine In one experiment (no 519) reserpine at a dose of

Table VI Experiments with alkaline urine

Expt no	Injected dose, μg leg vein		Bicarbonate wing vein	ATEF 100		Recovery per cent of dose		pH of urine (range)	Inj time (min)
	5-HT	Ph Red		5-HT	Ph Red	5 HT	Ph. Red		
462	200	800	–	23 0	31 7	25.5	75 8	5.5–5 7	3
	200	800	10 mg/kg/ min	39.2	38 0	41 7	92 9	6 4–7 7	3
464	200	800	–	14 7	19.9	20 0	71.3	5 4–5 7	3
	200	800	10 mg/kg/ min + 5 mg/ kg Diamox	13 6	11.5	18 0	71.2	7.5–8 0	3

7 5 mg was injected into one leg vein just before the injection of serotonin phenol red Not even here any reserpine induced inhibition of serotonin or phenol red transport could be observed (Table V B)

8 *Experiments with alkaline urine*

By infusion of isotonic bicarbonate into the wing vein it was possible to attain a neutral or slightly alkaline urine The pH of the diluted chicken urine which normally was about pH 5 5 was increased to between pH 6 4—7 7 after infusion of isotonic bicarbonate In one experiment where also 5 mg/kg acetazolamide was injected systemically together with the bicarbonate the pH of the diluted chicken urine went up to pH 8 0 The results (see Table VI) did not suggest any pH dependence of either serotonin or phenol red transport No attempt was made to collect the urine under anaerobic conditions This was also considered unnecessary by LINDAHL and SPERBER (1958) since the buffering capacity of the uric acid was considered to be rather high

Discussion

Serotonin is most probably excreted by the same transport mechanism as are many bases The influence of tolazoline consistently shows a competitive inhibition of the serotonin excretion The blood flow of the kidney was probably not decreased when the substances were injected into the leg vein as phenol red excretion was not depressed An attempt was made to inhibit the transport mechanism by giving tolazoline systemically as with probenecid This did not give any effect on the renal transport at a dose of 10 mg/kg, or possibly promoted an increase of serotonin transport This was probably due to the fast excretion of tolazoline as has been shown in man and in the dog by BRODIE ARONOW and

ASTERON (1952) When however 50 mg/kg tolazoline was injected systemically both serotonin and phenol red excretion were decreased probably as a reflection of vasomotor disturbances or possibly a toxic effect on the renal tubules These experiments in a way illustrate the superiority of the Sperber technique where substances can be injected almost directly into the intertubular capillaries.

Probenecid promoted a strong inhibition of phenol red transport after a wing vein injection 1 hour previously but left the serotonin excretion unchanged When tolazoline was here co-administered in the leg vein both phenol red and serotonin transport were inhibited

Brom cresol green could also inhibit phenol red excretion but the excretion of serotonin was obviously not influenced

The cyanine dye experiments pointed to a tubular inhibition of the base transport but this inhibition seemed to be weak and of low specificity since the higher doses of cyanine also inhibited the transport of phenol red probably by interference with the cellular metabolism of the renal tubules

If there really were a transport system sensitive to previous treatment with reserpine the ATI I values for serotonin in these pre treated birds would be smaller than in non reserpinized tolazoline controls This was not apparent On the contrary the inhibition by tolazoline was here if anything somewhat less than in the non reserpinized birds

When reserpine was injected into the leg vein together with serotonin or just before no inhibition of serotonin transport could be observed

Thus reserpine did not inhibit the serotonin transport

The tubular excretion of substances may be influenced by the pH of the urine using a non ionic diffusion of bases from the neutral peritubular blood into the acid tubular lumen (KESTERON 1939)

The present results did not indicate any pH dependence on the renal tubular excretion of serotonin in the chicken

Thus serotonin is transported in the chicken tubule by the ordinary organic base system

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Studies on the Reactions of the Cutaneous Vessels to Cold Exposure

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Abstract

Folkow, B., R. H. Fox, J. Krog, H. Odelram and O. Thorén. *Studies on the reactions of the cutaneous vessels to cold exposure.* Acta physiol. scand. 1963. *58.* 342—354. — Some inter-relationships between the mechanisms responsible for the vascular responses to intense cooling have been studied in cats and in man. The results suggest that both the *initial intense blood flow decrease* and *the subsequent cold vasodilatation* are complex in nature, each appearing to depend on a number of quite different mechanisms which are briefly discussed. There are good reasons to assume that the relative importance of each of the mechanisms involved may vary considerably depending on the circumstances, and that under certain conditions one or more may even be eliminated without changing the trend of the net response.

It is a well known phenomenon that skin vessels first exhibit a constriction when exposed to cold with a subsequent vascular relaxation — the so called cold vasodilatation — if the chilling is intense enough. The mechanism for these vascular reactions is still far from clear, though a number of studies have contributed much to the elucidation of some of the factors involved. There is evidence indicating that the reaction may in fact be very complex, involving a number of factors which are sometimes additive in their effects and sometimes counteract each other. In the present experiments, briefly reported elsewhere (Folkow *et al.* 1960), an attempt has been made to analyse the inter-relationships of some of the factors involved in the initial vasoconstrictor response and the subsequent cold vasodilatation both in the paw of the cat and in the hand of man.

Methods

Altogether 15 experiments were performed on cats The animals were anesthetized with chloralose 60 mg/kg and urethane 100 mg/kg after induction with ether Blood pressure was measured in one of the carotid or brachial arteries For the study of the reactions of the skin vessels the blood flow through one of the hind paws was measured For this purpose the big saphenal vein was exposed at the level of the ankle joint Other cutaneous veins at this level of the limb were ligated in order to direct as much as possible of the venous drainage of the paw to the big saphenal vein After heparinization, the big saphenal vein was ligated and cannulated in its distal direction The venous outflow from the paw was then led to a closed optical drop recorder operating an ordinate writer From the drop recorder the blood was returned to the animal by the cannulated central end of the saphenal vein or by the way of the femoral vein of the other limb

The saphenal and the sciatic nerves containing the great majority of the vasoconstrictor fibres to the vascular area studied were cautiously dissected free above the knee joint Thin ligatures were placed loosely around the nerves so that the paw vessels could if needed be easily denervated in the course of the experiment by cutting the nerves In a number of experiments in which direct stimulations of the vasoconstrictor nerves were performed together with intra arterial injections of vaso-active drugs the abdomen was open d in the midline and the big and the small intestines were extirpated leaving the stomach, the duodenum and other abdominal organs intact A cannula was inserted in the central end of the inferior mesenteric artery for i a injections The abdominal sympathetic trunks were then exposed at a level of L3—L5 and in some of the experiments the trunks were left intact to start with In other experiments the sympathetic trunk supplying the limb was centrally cut cautiously exposed and freed to allow direct graded stimulations of the vasoconstrictor fibres at a level of L4—L5 where all preganglionic fibres have reached the sympathetic trunk but where practically all the fibres to the paw are still present Square wave electrical stimulations were performed using a Grass stimulator model S4C Usually a strength of 4—5 volts and a pulse duration of 5 msec were utilized which in earlier experiments have been found suitable to induce an excitation of all the sympathetic fibres contained in the nerve trunk. The frequencies used varied betw een one impulse each 4th second and 10 impulses per second

To allow rapid cooling of the paw a small water bath containing stirred ice water was so arranged that the paw could be immersed In some experiments the paw was simply embedded in cotton wool abundantly soaked with a cold mixture composed of a saline solution just below 0 C A thermometer was placed near the paw and the cooling solution was repeatedly added to the cotton wool surrounding the paw to keep its temperature close to zero degrees The arrangement was found to induce effective cooling To measure the tissue temperature of the paw a needle thermocouple was inserted in the subcutaneous tissues between two of the pads and the tissue temperature was recorded continuously by means of a sensitive galvanometer

In some of the experiments arrangements were made to allow cooling not only from the outside of the paw but also cooling of the arterial inflow The femoral artery was then divided and its two ends connected via cannulas with the ends of a thin walled metal tubing with a big surface area and a low flow resistance which was immersed in a cooling bath. The arterial blood flow to the limb could then be effectively cooled down towards zero degrees and its temperature measured by a needle thermocouple inserted into the tubing close to the distal end of the artery The cold venous return after having passed the drop recorder was led through a similar device immersed in stirred water at a temperature of about 40 C. The rewarmed venous blood was

then returned to the animal by a suitable vein. Using this preparation the reactions of the paw vessels were compared with those of the calf muscles in order to reveal possible differences in the vascular reactions of the two tissues to cold. When muscle blood flow alone was to be measured the calf muscles were cautiously skinned after the paw had been amputated. The muscles were then soaked with Tyrode solution and protected against drying by a thin rubber membrane. The outside cooling was performed by way of ice water-soaked cotton wool as described above. The temperature of the muscle tissue was measured by a needle thermocouple inserted deep into the calf. Because of the fairly big muscle mass cooling the calf took longer than the paw even though the arterial inflow was also cooled.

Twenty-nine human experiments were performed on 12 young healthy subjects, applying the conventional venous occlusion plethysmographic method for recording hand blood flow. For this purpose two hand plethysmographs were used in which any desired water temperature surrounding the hands could be maintained over the range of 10—45 C. For the lowest temperatures the plethysmographs were filled rapidly with ice water. In this way the temperature could be dropped suddenly to about 2—3 C and then maintained.

The subjects rested comfortably in a reclining posture and were initially warmed with blankets in a room at 23—24 C, so as to reduce the initial sympathetic vasoconstrictor fibre activity in the skin to very low levels. The blood flow in the left hand was used as a control and to detect any generalized reflex vasomotor changes induced by exposing the other hand to ice water. The plethysmograph temperature was kept around 35 C throughout the experiment. Locally induced vascular reactions and the superimposed reflex vasomotor adjustments produced by rapidly lowering the temperature to 2—3 C were measured in the right hand after a control period during which the plethysmograph temperature had been kept at 35 C. With this technique it was possible to follow both the immediate vasoconstrictor response to cooling and also the subsequent cold vasodilatation. The effects of vaso-active drugs, nerve blocking and histamine blocking substances on the vascular reactions to cold in the right hand were studied by administering the substances to the hand through a catheter introduced into the corresponding brachial artery. In this way it was possible to study the changes in vascular reactions to injections of noradrenaline before and during cooling and also to investigate to what extent nerve blocking agents like xylocaine or histamine blocking agents like neoantergan affected the development of the initial vasoconstrictor response and the subsequent cold vasodilatation.

Results

Experiments on cats

The development of the initial vasoconstriction and the subsequent cold vasodilatation. In practically every experiment it was observed that the result of ice water cooling was a constriction of the paw vessels; this was usually slower in developing than in man. The vasoconstriction was found both in limbs where the sympathetic vasoconstrictor fibres were intact and in acutely denervated limbs (see Fig. 1). The extent of this initial vasoconstriction was generally not very different in the two limbs in anesthetized animals. It should be remembered, however, that anesthesia is known to interfere with the hypothalamic temperature control and therefore probably also with such reflex adjustments of the skin vessels that are relayed at the hypothalamic level. In the experi-

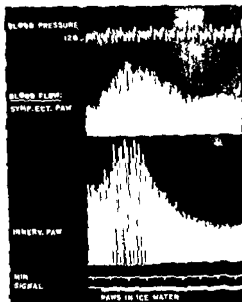


Fig 1 Anesthetized cat 3 5 kg A comparison of the vascular responses to intense local cooling in an acutely sympathectomized and a normally innervated hind paw Note that the initial vasoconstrictor response is not larger in the normally innervated paw indicating that little or no *reflex* vasoconstriction is induced in the anesthetized animal. Due to the somewhat more pronounced initial tone of the normally innervated vessels the subsequent flow increase is relatively more marked though the absolute flow level reached at the peak of cold vasodilatation is not bigger

ments on man described below there was generally evidence of a pronounced reflex adjustment superimposed upon the locally released vasoconstriction this is illustrated in Fig 4 After 3—6 min the paw vessels relaxed and a more or less pronounced cold vasodilatation was generally seen The vasodilatation developed relatively slowly and never with the striking suddenness sometimes seen in the human hand In some experiments a typical though small hunting reaction was also observed However once the cold vasodilatation was established prolonged periods of intense vasoconstriction never recurred in the cat The extent of flow increase in the cold vasodilatation was generally most pronounced in the experiments in which the initial vascular tone was high probably due to a tonic vasoconstrictor fibre influence (see Fig 1) although the level of blood flow at the peak of vasodilatation was then also somewhat lower Cooling the arterial inflow did not produce any obvious qualitative difference in response except that the initial vasoconstriction was more intense and sometimes more prolonged

The blood vessels of the calf muscle also responded with an initial vasoconstriction to cooling and in some experiments a definite vasodilatation followed after 10—20 min this was also observed after the blood vessels had been acutely denervated The increase in muscle blood flow was however only of the order of 25—50 % and therefore very small compared with the 3—5 fold increases that can be induced at normal tissue temperature by intra arterial injections of a vasodilator agent such as acetylcholine Nevertheless the vascular relaxation and widening with cooling is probably far greater than th

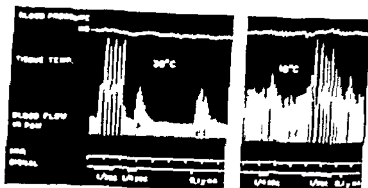


Fig. 2. Anesthetized cat 3 kg. A comparison of vasoconstrictor effects caused by low frequency constrictor fibre stimulation and by intra-arterial injection of noradrenaline at 30° C and 10° C local tissue temperature. Note that the responses to constrictor fibre stimulation and to noradrenaline injection are reduced to about the same extent by tissue cooling.

blood changes indicate because cooling also increases the viscosity of the blood to a considerable extent. This factor must also be considered when evaluating the extent of the cold vasodilatation in the skin.

Thus these experiments indicate, as could be expected, that the skin of the cat's paw and, to a lesser extent, the muscle blood vessels initially constrict and later dilate in response to cooling both when the nerves to the region are intact and also when the preparation is acutely denervated. In the paw these reactions can be intense and fluctuations in flow indicating the hunting phenomenon (Lewis 1930) have been observed.

The reactions of the blood vessels to vaso-active drugs and to constrictor fibre stimulation at different temperatures. Figure 2 illustrates an experiment in which the blood flow from one of the hind paws was recorded and the effects of direct electrical stimulation of the constrictor fibres and i.a. injections of vaso-active drugs were studied. In the first part of the experiment shown in Fig. 2, when the paw temperature was about 30° C, even low frequency stimulation induced powerful constrictor responses, as was also the case when 0.1 µg noradrenaline was injected i.a. It is seen from Fig. 2 that later in the experiment, when the paw had been *gradually cooled to 10° C by external cooling, both the effects* of sympathetic stimulation and injected noradrenaline were markedly reduced in extent and to about the same degree. This approximately equal reduction in neurogenically induced and blood borne catecholamine vasoconstriction was the usual finding. With paw temperature a few degrees above 0° C the response was sometimes only 1/10th to 1/20th of the normal, very sluggish in onset and retarded in relaxation. The response to vasodilator drugs like acetylcholine were similarly reduced. A considerable reduction in these responses was already evident when the paw was cooled to 20° C but the reduction only became very pronounced at temperatures below 10° C (see Fig. 3).

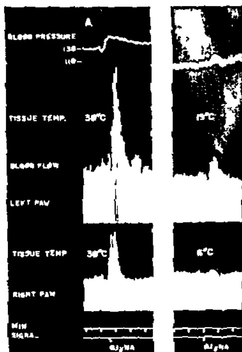


Fig 3 Anesthetized cat 3.2 kg Illustration of the extent of reduction of the vasoconstrictor effects of intra arterially injected noradrenaline when the paw temperature is reduced from 30 C to 19 C respectively

Experiments on man

In the experiments on man reactions to cooling were studied in one hand while using the opposite hand as a control In contrast with the animal experiments general anesthesia was not used so that reflex vascular adjustments from the higher centres of the central nervous system should appear In the first series of experiments the occurrence and the extent of a generalized reflex cutaneous vasoconstriction was studied After a series of initial control readings the water at 35 C in the right hand plethysmograph was rapidly exchanged with water at 0 C and recordings of blood flow in both hands continued The intense cooling caused a rapidly developing vasoconstriction which reduced the blood flow in the right hand from some 15 ml/100 ml of tissue/min to about 1—2 ml/100 ml of tissue/min In the left hand where the temperature was kept constant at 35 C blood flow was also reduced although less markedly as the result of reflex vasoconstriction The much larger vasoconstriction observed in the cooled hand must therefore be ascribed to the combined effects of a local cold induced vasoconstriction presumably superimposed on a reflex neurogenic component as in the control hand These effects are illustrated in Fig 4 which is based on the mean values obtained from four subjects The reflex vasoconstriction in the control hand generally disappeared within 3

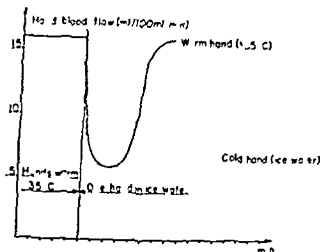


Fig 4 Diagram based on the mean values from four subjects illustrating the blood flow changes in the two hands when one hand is suddenly exposed to ice water while the other hand is maintained in a local temperature of 35 C

min Vasoconstriction in the cooled hand remained very intense for about 6—15 min after which the typical fluctuations in flow of cold induced vasodilatation were seen This is in agreement with several earlier studies in man (cf KROG et al 1960)

Two procedures were used to study constrictor fibre influence in the cooled hand In a number of experiments noradrenaline was given by i a injection both when the hand was warm and when it was cooled As in the animal experiments the response to a given amount of noradrenaline was reduced some 10 to 20 times when the hand temperature was lowered from 30—35 C to 2—6 C It can therefore be expected that in the human hand as in the cat's paw the influence of a given degree of constrictor fibre activity will be considerably reduced with cooling The importance of the reduction of the constrictor fibre influence on the vessels at lowered tissue temperature was further demonstrated by comparing the results of cooling the hand from 35 C to 2—6 C when the subject was feeling warm and when the subject was feeling cool In the control period before local cooling the hand blood flows of the warm subjects were some 10 to 20 times higher than when the same subject was feeling cold due to the difference in vasoconstrictor fibre activity within the skin With local cooling of the hand, blood flows decreased much more when the subject was warm than when cooled, so that shortly after cooling and also during the ensuing cold vasodilatation the hand blood flows in the warm subjects were only about 2—3 fold greater than in the cool subjects This is illustrated in Fig 5 which is based on the mean values from nine subjects

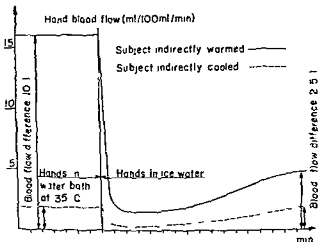


Fig 5 Diagram based on the mean values from nine subjects illustrating the considerably reduced though still evident vasoconstrictor fibre influence on the hand blood vessels in an environment of ice water as compared with an environmental temperature in the plethysmograph of 35 C The reduced influence of the constrictor fibres is evident from the shift in the flow ratios relating the indirectly warmed (low constrictor fibre activity) and indirectly cooled subjects (high constrictor fibre activity) when the local tissue temperature is lowered

These results indicate as did the vascular responses to noradrenaline injections that the vascular responsiveness to vasoconstrictor influence is greatly reduced though not entirely abolished when the local tissue temperature is lowered enough to cause a cold induced vasodilatation

In a further series of experiments designed to investigate the role of local nervous factors such as axon reflexes in the cold induced vasodilatation response the right hand was exposed to icewater in the usual way before and after a local anesthetic (xylocaine) had been injected i a Attempts were made to inject enough xylocaine to render the hand insensitive to pain and temperature for at least 10—15 min To increase the absorption of the anesthetic in the hand an arterial cuff was inflated immediately after the injection After about 5 min the cuff was released and the vascular reaction to cooling the hand were studied Despite the precautions taken it proved very difficult to prevent the rapid elimination of the anesthetic agent but in a few experiments the anesthesia was so pronounced that the subject felt neither cold nor pain on cooling the hand In these experiments the axon reflexes connected to the pain fibres and probably also the vasoconstrictor fibres should have been more or less completely blocked Nevertheless cold vasodilatation ensued although the extent and the rate of development of the vasodilatation was not as big as in the unanesthetized hand In other experiments i a infusion of an antihistaminic agent (promethazine chloride Lergigan) in amounts sufficient to abolish the vascular effects of histamine were given There evidence that this procedure decreased the cold vasodilatation

Discussion

Intense cooling of the cat's paw induced peripheral blood flow responses which in principle were closely similar to those observed during cooling of the human hand. There was an initial vasoconstriction usually followed by vasodilatation. Alternating periods of vasoconstriction and vasodilatation, the "hunting phenomenon", typical of cold vasodilatation in the hand (LEWIS 1930) although sometimes seen, were never a marked feature of the response in the paw. Frequently the initial vasoconstriction was followed by a steady and sustained vasodilatation. The response was still present, though less brisk, in the sympathectomized paw, as it is in the sympathectomized hand (LEWIS 1930).

It has been suggested that a failure of conduction in vasoconstrictor nerves might explain the phenomenon of cold induced vasodilatation (HERTZMAN and ROTH 1942). In general, however, unmedullated fibres are among the last nerves to be affected by cooling (DOUGLAS and MALCOLM 1955) and conduction has been demonstrated at 0° C (LUNDBERG 1948). It seems clear that the vasoconstrictor fibres can affect the vessels, at least to some extent, even at very low tissue temperature, because the cold vasodilatation obtained in man is considerably reduced by general body chilling (LEWIS 1930, DUFF *et al.* 1953, KEATINGE 1957), as was also observed in the present experiments (*e. g.* Fig. 5). It is clear, however, that the magnitude of a given vasoconstrictor fibre effect is strongly reduced by local cooling, which is demonstrated in the present experiments both in the cat and in man. On the other hand, the vasoconstrictor responses to i. a. injected noradrenaline were reduced to approximately the same extent, which points to a decreased responsiveness of the vascular smooth muscle cells rather than to any failure of nervous transmission or of transmitter release. A similar failure in responsiveness to vasoconstrictor agents has been shown in *in vitro* studies on segments of the ulnar arteries of bullocks when temperature is reduced below 10° C (KEATINGE 1958).

A reduced sensitivity of the vessels to catecholamines when cooled may therefore be considered to contribute to the cold vasodilatation, whenever a tonic constrictor fibre activity is present. The constrictor fibres appear to contribute to the initial vasoconstrictor response also, as a brief reflex intensification of their activity is easily demonstrated upon local cooling.

Other factors must also contribute both to the initial vasoconstriction and to the subsequent cold vasodilatation, because both phases could still be observed when all vasoconstrictor fibres to the cat's paw were cut and the adrenal glands denervated or extirpated or when a local anesthetic was administered intra-arterially. There is clear evidence in the literature that axon reflexes from thin afferent fibres — presumably pain fibres — are of considerable importance for inducing cold vasodilatation, at least in the skin (LEWIS 1927, WYBAUW 1936, KRAMER and SCHULZE 1948, CELANDER and FOLKOW 1953). Although KRAMER and SCHULZE (1948) were unable to demonstrate any cold vasodilatation after infiltration of the pulp with a local anesthetic, which would

block both the vasoconstrictor fibres and any axon reflex mechanisms GREENFIELD SHEPHERD and WHELAN (1952) found that a reduced but not insignificant cold vasodilatation could still be induced under such circumstances The present experiments are in agreement with the results of the latter group of investigators as some cold vasodilatation was still obtained after the hand was temporarily anesthetized by xylocaine 1 % given in concentrations sufficient to prevent any pain or cold sensations on immersion in ice water The presence of still another mechanism beside the interference with the constrictor fibre control and the activation of axon reflexes is further suggested by the fact that some cold vasodilatation could be elicited in acutely sympathectomized skeletal muscles where blood borne catechol amines were ruled out Here the cold vasodilatation cannot be ascribed to any axon reflexes connected to thin afferent fibres as in this tissue — in striking contrast to the cutaneous vascular region — no such axon reflex mechanisms can be demonstrated even on intense antidromic excitation of the afferent nerve fibres (CELANDER and FOLKOW 1953) It is known on the other hand that the resistance vessels of the skeletal muscles exhibit a pronounced tone when denervated and deprived of any extrinsic vaso excitatory agents and in all probability this tone is of a truly myogenic origin (e g FOLKOW and ÖBERG 1961) It is not unreasonable to assume that this inherent smooth muscle activity like most activities of living cells will be considerably depressed or even abolished when the tissue temperature is reduced to low levels producing a vasodilatation It is true that the observed flow increase in the skeletal muscle upon cooling was generally relatively small at least when compared to the four or fivefold flow increase that can be induced by 1 a injected acetylcholine at normal tissue temperature It should however be realized that blood viscosity is markedly increased upon cooling (e g NICHOL 1952) and erythrocyte aggregation sludging tends to occur factors which *per se* will strongly reduce the rate of blood flow Therefore, although the blood flow may not be high the actual vascular relaxation may nevertheless be profound Increasing vascular relaxation through inhibition of myogenic activity as the local temperature is lowered will in turn tend to be counteracted to some extent by yet another factor — the reduction in vasodilator metabolites It is known that vascular tone in most regions is sensitively adjusted to meet the needs of tissue metabolism With cooling tissue metabolism is depressed and less metabolites are produced which — other things being constant — will tend to enhance vascular tone This factor is probably not quantitatively very important but must nevertheless be considered

Finally it is known that severe tissue damage can sometimes occur as the result of quite short exposure to temperatures several degrees above zero (EDHOLM *et al* 1957) also two instances of localized sensory nerve degeneration following immersion of the hand and forearm in water at 0 C for 1/2 hr have been observed (FOX and KROG unpublished)

It is therefore not improbable that cooling to a few degrees above zero does represent a noxious stimulus capable of causing the early changes of tissue damage. Tissue damage is known to be accompanied by the release of vasodilator compounds — the H substance of LEWIS (1927). It is unlikely that histamine itself is the important vasodilator compound, because antihistamines were found to be without any effect on the cold vasodilatation in skin and in skeletal muscles in this study, in agreement with the findings of DUFF *et al.* (1953). LEWIS recognized that H substance was a complex material and since one of its components was found to be relatively non-diffusable KROG (1929) suggested the name H colloid. Recently it has been shown that bradykinin, which is a powerful vasodilator administered i. a. to man (FOX *et al.* 1961), can produce all the cardinal signs of local inflammation and that this polypeptide or the enzyme producing it may correspond to H colloid itself (LEWIS 1961). Final elucidation of a possible role of bradykinin in the cold vasodilatation phenomenon will probably have to await the development of specific inhibitors of its production or destruction.

The fact that cold vasodilatation can also to some extent be induced in a tissue like skeletal muscle, where no a-v shunt vessels have been demonstrated, is of interest from another point of view, as it has been claimed that the increased flow of cold induced vasodilatation passes through a-v anastomoses (GRANT 1930, GRANT and BLAND 1931). Cold vasodilatation in the human forearm, also believed to be muscular in origin, has been described (CLARKE, HELLON and LIND 1957) and weak vasodilator responses have been elicited from many skin areas in man which do not exhibit the typical marked cold vasodilatation phenomenon and probably do not have a-v anastomoses (FOX and WYATT 1962). No doubt the biggest cold vasodilator responses are seen in cutaneous regions rich in a-v shunts, but the difference is probably more quantitative than qualitative in nature.

To summarize this discussion, the present experiments are in good agreement with previous studies and taken together they suggest that both the initial blood flow decrease as a response to local cooling and the subsequent cold vasodilatation are produced by fairly complex co-ordinations of several quite different mechanisms. There are reasons to believe that the relative importance of the individual mechanism can vary depending on the particular circumstances and that certain of them may sometimes be absent without changing the trend of the net response.

At least four factors may be considered to cooperate in the creation of the initial blood flow decrease. A dominating feature appears to be the reflex *excitation* of the vasoconstrictor fibres, a neurogenic constrictor response that is superimposed upon a likewise powerful but direct constrictor response of the vascular smooth muscles which is presumably an excitatory effect of the drastic temperature fall. A direct constrictor effect produced by rapid cooling has been observed in several types of smooth muscles (PERKINS *et al.* 1950)

including isolated blood vessels (SMITH 1952) Although initially powerful both these influences on vascular tone tend to be fairly limited in duration The increased blood viscosity caused by the cooling is however a lasting effect which adds to the decrease in blood flow, in proportion to the actual viscosity of the blood without any change in the vascular dimensions In addition tissue cooling lowers the metabolism of the local tissue and therefore reduces the formation of vasodilator metabolites in the immediate environment of the vascular smooth muscles a local chemical change which *per se* tends to increase their tone

If the temperature fall is sufficiently drastic and sufficiently prolonged the factors promoting a decrease in flow through vasoconstriction may be overcome by other factors tending to cause vasodilatation In the skin axon reflexes from the abundant supply of thin pain fibres appear to play some part Possibly more important at least in situations of a tonic vasoconstrictor fibre activity, cooling markedly depresses the sensitivity of the vascular smooth muscles to the released adrenergic transmitter which will reduce — but not abolish — the vascular influence of these fibres Furthermore cooling below 10 C appears to cause a progressive depression in the myogenic activity or 'inherent tone of the resistance vessels which if sufficiently marked must lead to a passive relaxation and vasodilatation Finally the local release and because of the low blood flow accumulation of specific vasodilator compounds especially those resulting from the early stages of tissue damage may play an important role and contribute to the establishment of the hunting reaction At low temperatures the reactivity of the vessels to such compounds is markedly depressed but once vasodilatation has been initiated possibly largely through passive relaxation the increased blood flow will raise the local temperature and they will then exert a progressively more powerful vasodilator influence However after a period of increased blood flow the release of these vasodilator compounds would diminish and already accumulated concentrations would be removed The same is probably true of the transmitter released at the axon reflex nerve endings Furthermore at the higher tissue temperature then prevailing the vasoconstrictor factors would again become effective and re establish vasoconstriction

In the light of our present knowledge such a system of multiple interacting and opposing forces which include the unstable qualities needed to explain the violent fluctuations in flow often seen in cold vasodilatation appears the most likely explanation for the phenomena observed

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Acta physiol scand 1963 58 355—358

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Intramyocardial Temperature Gradients in Dogs During Hypothermia by Surface Cooling

By

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Received 17 December 1962

Abstract

JOHANSSON B W *Intramyocardial temperature gradients in dogs during hypothermia by surface cooling* Acta physiol scand 1963 58 355—358 — *In five* dogs the temperature in different parts and at different depths in the myocardium *were* recorded during cooling till asystole *or* ventricular fibrillation appeared It was *found* that the temperature gradients were small and that there was *no* increase in the gradients with decreasing temperature *It is* concluded that the ST T changes observed during hypothermia *in* homeothermic animals are *not* caused by intramyocardial temperature gradients

Profound ECG changes appear during hypothermia in animals and man (BJÖRCK and JOHANSSON 1955 HAEGER JOHANSSON and SJÖSTRÖM 1957 JOHANSSON *et al* 1956) These changes can be ascribed both to a direct effect of the low temperature (decrease of heart rate and prolongation of the different time intervals) and to more indirect effects of the hypothermia mainly changes in the ST T complex The cause of the latter changes have been discussed but no conclusive results have appeared (JOHANSSON *et al* 1956) It is known that temperature gradients *in* the myocardium can produce changes in the ST T complex (HELLERSTEIN and LIEBOW 1950) T wave changes after drinking iced water have been reported by *for* example DOWLING and HELLERSTEIN (1951) The occurrence of profound temperature gradients within the body during cooling was shown by VAN DER HEIDE 1956 GELL *et al* 1956 SELLICK 1957 BJÖRK 1960

It is thus conceivable that *the* blood coming from the peripheral vessels *to* the right atrium and ventricle might have a temperature low enough to create temperature gradients in *the myocardium* and that these gradients *might* at least be contributing factors to the ST T changes *during* hypothermia

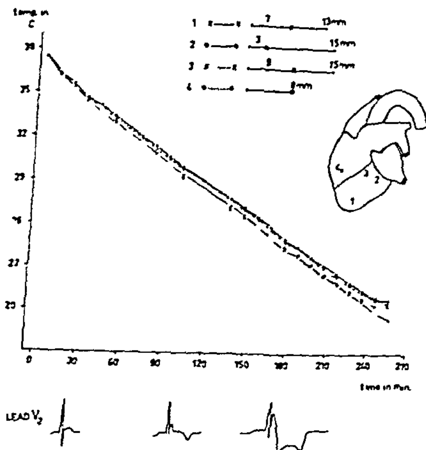


Fig. 1. Temperature recordings from different parts of the heart of a dog during cooling. 1, 2 and 3 refer to different parts of the left ventricle and 4 to the right ventricle. At point 1 13 mm means that the ventricular wall was 13 mm thick at this point. The x means the point where the tip of the thermocouple electrode was situated. At point 1 the tip thus was 7 mm below the epicardial surface. The tip of the R wave indicates the temperature and the time after the beginning of cooling that the ECG was recorded.

Material and methods

Five mongrel dogs with no signs of disease weighing between 15 and 31 kg and of an age varying between 5 months and 4 years were anesthetized with pentothal until both the medial and lateral lid reflexes disappeared. The dogs were intubated and artificial respiration with air was given during the whole experiment. A left thoracotomy was performed using a clean but not sterile technique. The pericardium was opened and four thermocouple electrodes were introduced through small stab wounds of varying depth into the myocardium of the left and right ventricles. They were kept in place with a purse string suture. In two experiments a fifth thermocouple electrode was introduced in the right and left auricular appendage respectively with the tip free in the atrial blood. The thermocouple allowed continuous recordings of temperature changes to within 0.1 C.

The pericardium was left partly open and the thorax — closed A ? was taken immediately after the closure and at a temperatur of 0 25 a direct writing equipment (Elema Mingograph) The animals were fngeration box as described by BIORCK et al (1954) After asystole or ver lation had appeared the heart was gently removed and the exact posi trodes in the myocardium was determined See the legend to Fig 1 for The duration of the refrigeration procedure varied between 3 3/4 and 4

Results and comments

The results of all 5 experiments of which Fig 1 is representative at in conformance with each other and show clearly that the temperature differ ences between the electrodes are small and that these differences are not in creased with decreasing temperature The experimental data do not supp rt the hypothesis that intramyocardial temperature gradients are the cause of the electrocardiographic changes observed during hypothermia

It might be argued that local bleeding and deteriorated blood circulation around the thermocouple electrodes might produce a false recording If this were the case the trauma would probably vary at the different electrodes thereby producing temperature gradients Furthermore in the dog with one electrode in the right atrium the exploration after the experiment revealed that the tip of the electrode was situated 5 cm down in the inferior vena cava and not even in this case were any temperature gradients found

The present results do not conform with those reported by FISHER and FEDOR (1961) who observed cardiac temperature gradients sometimes very pronounced during hypothermia However these authors used another experimenta technique with extracorporeal circulation and heat exchanger which resulted in a much more rapid cooling (from normothermia to 5—10 C in abou 30 min) than in the present experiments FISHER and FEDOR (1961) concluded that thermal gradients alone are not responsible for hypothermic ventricular fibrillation This conclusion is supported by the present study in which 3 animals fibrillated without showing temperature patterns differing from the 2 animals that did not fibrillate

The excellent technical assistance of Miss B ARRO EKLUND is gratefully acknowledged
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Acta physiol scand 1963 58 359—367

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Lactate Content in Sweat

By

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Abstract

Åstrand I *Lactate content in sweat* Acta physiol scand 1963 58 359—367 — Four subjects were heated by means of physical exercise in different room temperatures or with hot baths in order to bring about sweating The sweat was collected in plastic bags from the extremities in at least 3 consecutive 15 min periods and analysed for the content of lactate and chloride In some experiments the blood circulation was occluded with a blood pressure cuff The lactate concentration was significantly higher during cycling than bathing and significantly higher during the first period as compared to the following The lactate concentration rose during occlusion of the circulation The secretion of lactate per minute was rectilinearly correlated to the sweating rate and to the chloride secretion The first period of cycling revealed however significantly different values from the others in this respect The results are discussed with regard to degradation of glycogen to lactate in the sweat glands to diffusion of lactate into the blood to reabsorption intensity of the sweating stimulus and to the content of lactate in the epidermis

The concentration of lactate in sweat is high especially in the beginning of a sweating period whether this is elicited by thermogenic stimulation of sweat glands or by heavy muscular exercise (Weiner and van Heyningen 1952) There is no acceptable explanation for these findings

In the present investigation short consecutive periods of sweat collection were chosen in order to analyse carefully the course of the concentration The relation of lactate content to sweating rate chloride content and to secretory work is discussed

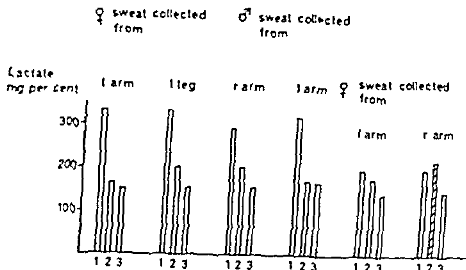


Fig 1 Lactate concentration in eccrine sweat from left arm and leg in a female subject during cycling from right and left arm in a male subject during cycling and in sweat simultaneously collected from left and right arm during bathing The sweat was collected from 0—15 min (1) 16—30 min (2) 31—45 min (3) The circulation to the right arm during bathing was occluded during the second period by the aid of a blood pressure cuff (0 mm Hg lined column)

Materials and methods

Two female and two male healthy students were used as subjects

In two of the subjects sweating was elicited by exposing them (1) to heat radiation and (2) to different levels of atmospheric temperature (21—42 C) in both cases in combination with continuous muscular work of a moderate intensity on a bicycle ergometer (300—600 kpm/min oxygen uptake 0 9—1 5 liters/min blood lactate concentration 9—28 mg per 100 ml of blood) In addition sweating was elicited for three subjects (3) at rest by general heating in a hot bath

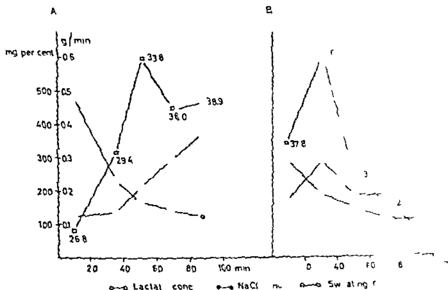
In order to vary the skin temperature locally in 9 exp the subjects submerged one arm enclosed in a plastic bag into water of different temperatures (19 0—45 3 C) The other arm was used as a control

In two experiments during bathing the circulation of one arm was occluded by a blood pressure cuff inflated to 220 mm Hg

Generally the sweat was collected from the arms only occasionally from the legs The whole extremity up to the middle of the upper arm and thigh respectively was thoroughly cleansed and afterwards rinsed with distilled water dried and enclosed in a plastic bag The sweating rate of an extremity was determined by weighing the collected amount of sweat The disadvantages of this method for the collection are discussed by VAN HEYNINGEN and WEINER (1952 a) ROBINSON and ROBINSON (1954) among others

The sweat was collected in consecutive periods of usually 15 min By experience it was found that the length of the periods could not be shorter because of the latency for the onset of sweating In some experiments in a lower atmospheric temperature and during bathing the first period was prolonged to 20 min Each experiment was continued for at least 45 min in some cases for 105 min

During the collection of the sweat the skin temperature of the extremity was measured on 3 places with thermo-couples usually every other minute When collecting from the



F g 2 Lactate concentrat on chloride concentration (calculated as NaCl an l sweat ng rat for one arm in different sweat ng periods The average skin temperature during a per od was varied by m ans of increasing (A) and decreasing (B ten perature in a l cal wat r bath

arms the measurements were made dorsally on the lower part of the upper arm a id on the middle of the forearm and hand respectively when collecting from the legs the measurements were made at comparable places The average skin temper ture of the extremity during one period was calculated as a mean value of all the meas ren nts

Fifty experiments were made 28 during muscular exercise and 22 at rest Not more than two experiments were made a week in an attempt to a oid a limatization to heat

The oxygen uptake was measured during the work experiments with the DOUGLAS bag method (the expired air was analysed with a HALDANE apparatus During these experiments the blood lactate concentration was determined

The lactate was analysed according to EDWARDS (1938) (the error of the method as ± 3 mg per cent for a double determ nation) and the chlorid with an electrometric microdetermination accord ng to LEHMANN (1933) (the error of the method was ~ 2 4 mg per cent calculated as NaCl for a do ible determination) The duplicates were made on different samples from the same period of sweating

Results

I *Lactate concentration*

There was no difference in lactate concentration betv een male and female subjects (cf OTTENSTEIN 1930) nor betv een the two arms nor bet een arms and legs (Fig 1)

The lactate concentration was higher in the sv eat from the first period as compared to the follov ing ones with only one exception i e in the experiments with the circulation occluded (Fig 1) In these last mentioned experiments the

Table 1 Mean values ± standard errors of the mean and S D of lactate concentration in sweat samples (see Methods) collected from 1—15 min (1st period) 16—30 min (2nd period) 31—45

Lactate conc during	1st period			2nd period	
	n	mg per cent	P	n	mg per cent
Cycling	28	338 ± 12.2 65	–	2	19[illegible] ± 6 1 3[illegible]
Bathing	2[illegible]	224 ± 8.1 38	< 0 001	2[illegible]	1[illegible]5 ± 4.5 2[illegible]

P = P value for the differences between ( ) 1st and 2nd 2nd and 3rd, 3rd and 4th period

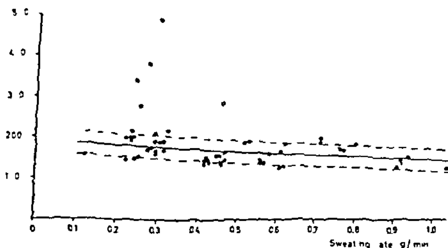


Fig 3 Lactate concentration in sweat in relation to sweating rate in two subjects. ‾ = cycling 1st period ● = cycling 2nd—6th periods and bathing 1st—3rd periods (n = 59) $\bar{x} \pm \epsilon x =$ *= 0 45 ± 0 03* $\bar{y} \pm \epsilon \bar{y}$ *= 173 ~ 4 0 regr eq y = 191—40 4 x corr coeff — 0.288 ± 0 122* deviation from regr line ± 30 (17 per cent)

concentration increased when the circulation was occluded However in the given case in Fig 1 it did not differ from the corresponding average values in experiments with normal circulation by more than 1 5 × S D

By changing the skin temperature during an experiment with the aid of water baths of different temperatures great variations were obtained in the sweating rate and the chloride concentration In these experiments the lactate concentration however varied only slightly after the first period (see Fig 2) nor was there any difference in this respect between the two arms

collected from the arms or the legs of four subjects during cycling and bathing The sweat was ... min (3rd period) and 46—60 min (4th period)

2nd period	3rd period			4th period		
P	n	mg per cent	P	n	mg per cent	P
0 01 > P > 0 001	28	165 ± 5 3 29	< 0 001	9	156 ± 5 2 16	> 0 1
0 0? > P > 0 01	18	137 ± 4 6 20	< 0 001	—	—	—

( ) between cycling and bathing in the same period

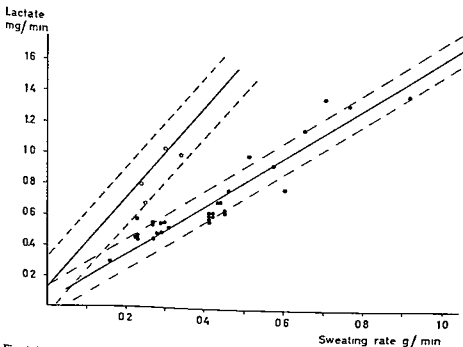


Fig 4 Lactate in mg secreted per min in relation to sweating rate in two subjects. ○ = cycling 1st period (n = 13) $\bar{x} \pm \epsilon\bar{x} = 0.25 \pm 0.03$ $\bar{y} \pm \epsilon\bar{y} = 0.87 \pm 0.11$ regr eq $y = 0.13 + 2.98\,x$ corr coeff 0.892 ± 0.062 deviation from regr line ± 0.18 (2? per cent) ● = cycling 2nd—6th periods and bathing 1st—3rd periods (n = 59) $\bar{x} \pm \epsilon\bar{x} = 0.45 \pm 0.03$ $\bar{y} \pm \epsilon\bar{y} = 0.75 \pm 0.05$ regr eq $y = 0.03 + 1.6\,x$ corr coeff 0.962 ± 0.010 deviation from regr line ± 0.10 (13 per cent)

For reasons given above all the data have been grouped according to collection period and then treated statistically (see Table I) During *cycling* the average concentration for the first period was 338 mg per cent with a higher

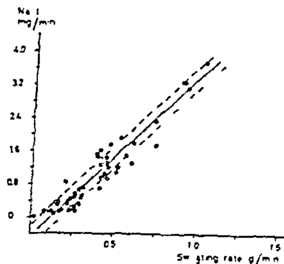


Fig 5 Chloride (calculated as NaCl) in mg secreted per min in relation to sweating rate during cycling in two subjects. = female subject ● = male subject n = 64 $\bar{x} \pm \sigma x$ = 0 39 ± 0 03 $\bar{y} \pm \sigma \bar{y}$ = 1 0? ± 0 05 regr eq y = — 0 5? + 3 94 x corr coeff 0 9?4 ± 0 012 deviation from regr line 0 ?8 (28 per cent)

value of 489 mg per cent for the second period it was 195 for the third 165 and for the forth 156 during *bathing* the corresponding values were 224 175 and 137 mg per cent respectively The significances of the differences between the periods are given in the table

The individual values for lactate concentration in relation to sweating rate were plotted in Fig 3 The data from the first period of cycling were significantly different from the values of all other periods including the values obtained in all experiments of bathing

II *Lactate secretion rate*

The secretion of lactate per minute during the first period of cycling was rectilinearly correlated to the sweating rate as were the values from all other periods but the level of secretion rate was significantly lower (see Fig 4) The standard deviation for the last mentioned regression line was relatively large partly due to the fact that the lactate secreted per minute was somewhat although not significantly higher in the second than in the third period and in the third as compared to the forth period

III *Lactate and chloride secretion*

The chloride secreted per minute was also rectilinearly correlated to the sweating rate (see Fig 5) Therefore the secretion of lactate per minute was correlated to the secretion of chloride per minute and the first period of cycling differed from the other periods

Data for the last mentioned correlations *cycling 1st period* regr eq y = 0 26 — 1 56 x corr coeff 0 914 ± 0 052 deviation from regr line ± 0 17 (20 per cent), *cycling 2nd—6th periods* regr eq y = 0 39 + 0 30 x corr coeff 0 818 ± 0 058 deviation from regr line ± 0 21 (29 per cent)

Discussion

Since the basic observation of SCHENK and WISSEMAN (1926) that the sweat contains a great amount of lactate its origin has been discussed many times. It is improbable that it derivates from the blood because of the great difference and variation in concentration between sweat and blood. Therefore it is more likely that lactate in sweat is produced by the skeletal muscles and of importance for the acid base balance of the body (cf. DILL 1938, p. 46, DILL et al. 1938).

Already in the beginning of this century BRUNNER (1906) among others could show the presence of glycogen in the eccrine sweat glands. (For literature see ROTHMAN and SCHAAF 1929, p. 268.) YUYAMA (1935) and SHELLEY and MESCON (1952) observed microscopically that the glycogen content decreases during sweating and reappears after a rest period. BUNTING et al. (1948) proved the presence of alkaline phosphatase and SHELLEY and MESCON (1952) the presence of acid phosphatase in the secretory tissue of the glands. Therefore it is easily assumed that the lactate in sweat originates from the degradation of glycogen to lactate and that the energy for the secretion is delivered anaerobically.

If the lactate in sweat derivates from the break down of glycogen providing the energy for the secretory work, its content should increase with increase in secretory work, which mainly consists of the excretion of chloride (WEINER and VAN HEYNINGEN 1952). As mentioned the secretion of chloride per minute increases with increasing sweating rate (cf. ROBINSON and ROBINSON 1954, ROTHMAN 1961, p. 213, and LOBITZ and DOBSON 1961). The secretory work ought therefore to be highest at the highest sweating rate, which in the present experiment occurred in the middle of or in the last stages of a sweating period of 1 hour.

After the first 15-min period in these experiments, however, the lactate concentration and content in sweat decreased. This is one of the difficulties involved when interpreting the role of lactate in a metabolic process essential for sweating. WEINER and VAN HEYNINGEN (1952), using several sweating periods of 1/2 hour, also found a similar but not so pronounced decrease. They also found an increase when the circulation was occluded (VAN HEYNINGEN and WEINER 1952b) and came to the conclusion that different degrees of anaerobic activity probably were prevailing in the sweat glands. DILL et al. (1938) doubted that anaerobic glycolysis on the whole should be found under normal conditions in the skin.

The lactate secretion per minute was significantly correlated to the simultaneous chloride secretion in the present study. This would be the case if the lactate originates from an anaerobic metabolic process whose intensity was proportional to the secretory work. Since ITOH et al. (1952) proved the presence of a higher concentration of pyruvate in sweat in the beginning of a sweating period than later on, such anaerobic conditions are even more probable.

However several phenomena can not be explained as simply (1) the different slopes of the regression lines for the lactate secreted per minute in relation to the sweating rate and to the chloride secretion per minute during the first period of cycling as compared to the other periods (cycling and bathing) (2) the different amounts of secreted lactate per minute in the 2nd as compared to the 3rd and in the 3rd as compared to the 4th periods (3) the increase in concentration when the circulation was occluded (even if the values do not significantly differ from the regression line in Fig 3) (4) the differences in the amounts of lactate secreted per minute in relation to sweating rate between the first period of cycling and bathing

However, some of the produced amounts of lactates may diffuse into the capillary blood In the beginning of exercise with constriction of the skin blood vessels relatively more lactate may disappear with the sweat As the vessels in the neighbourhood of the sweat gland dilate more lactate may diffuse into the blood and the gradual decrease in concentration of lactate in sweat could thus be explained as also the difference between the cycling and bathing values Prevention of the supply of fresh blood by occlusion of the brachial artery should increase the concentration of lactate in sweat which was also noticed

It is however also probable that a reabsorption of the sweat takes place in the ducts (VAN LLOYD 1959 THOMSON 1960) If especially water from the sweat is reabsorbed the lactate must remain in the duct after a period of sweating simulating a greater production of lactate in the beginning compared to later on during a sweating period If besides this the number of the functioning glands increases with increase in intensity of a stimulus (KUNO 1956 p 291 RANDALL 1946) the above mentioned *items 1—2* can be satisfactorily explained Number 4 may then accordingly be explained by a less intensive stimulus during bathing as compared to during cycling

Another explanation might be that lactate (pyruvate) found in the epidermis (LAHRIG 1927, PILLSBURY 1931) is partly washed out in the beginning of a sweating period simulating a high production in the sweat glands

The answers to all these questions cannot be found in these experiments They probably lie in a combination of physiological and histo-chemical studies of the sweat glands

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Acta physiol scand 1963 58 368—375

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Reversible Blood-Brain Barrier Alteration Induced by Certain Organic Acids and Indicated by Means of EEG and Dye Tests

By

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Abstract

FLODMARK S and O STEINWALL. *Reversible blood brain barrier alteration induced by certain organic acids and indicated by means of EEG and dye tests* Acta physiol scand 1963 58 368—375 — With an earlier described method for studies of the interrelation between EEG and blood brain barrier functions alteration of the barrier was induced by intracarotidal application of certain types of organic acids (represented by penicillin G and Urokon) In long term experiments it could be established that this type of barrier alteration was reversible as judged by intravital dye tests and further by EEG activating tests applied in the early and late phases of the experiments The EEG changes evoked by the invasion of normally barred organic acids into the barrier-damaged hemisphere were of an excitatory type and sometimes caused a convulsion pattern lasting for hours Even after such a heavy stress on the neurons however there was a reversal in the EEG sometimes to full normalization The EEG course after this reversible barrier alteration is different from that seen after the persistent barrier dysfunction induced by mercuric ions as reported in a previous paper The reversible action of the organic acids on the barrier is discussed as being compatible with the hypothesis previously proposed by Steinwall that brain blood directed transport mechanisms participate in the barring of this group of acid compounds

As part of a study on the relationship between blood brain barrier functions and EEG we recently investigated the influences on the EEG after a persistent barrier injury induced by a heavy metal (mercuric dichloride) which was applied within the vessels of one hemisphere under careful dosage control (FLOD

MARK and STEINWALL 1963 for methodological consideration see FLODMARK and STEINWALL 1962) For a limited period of time it w i p sible to obtain a state of barrier dysfunction (with abnormal penetration f rious dyes and related organic anions) without significant influence on the FI C l lima tely however the EEG deteriorated on the barrier damaged side re i m? owing to cumulative disturbances in the metabolic milieu of the i r ı ı I was proposed that the heavy metal under the experimental condit ns em ployed due to its strong tissue affinity was held within the barrier structur interposed between blood and neurons and there exerted a poorly reversible toxic action

In the present investigation the aim was to study with the same technique another type of barrier alteration induced by organic acids with a polar (hydrophilic) and a non polar (lipophilic) molecular configuration When applied within the cerebral vessels in adequate concentration in loco these agents induce blood brain barrier impairment while on the other hand the same acids under normal barrier conditions are unable to pass from blood to brain when administered into the general circulation (see STEINWALL 1961 FLODMARK and STEINWALL 1962) *On theoretical ground this type of barrier* dysfunction — unlike that induced by heavy metal ions — was presumed to be transitory in nature as was already stated experimentally by BROMAN and OLSSON (1948) concerning one representative of this group of acids (iodopyra cet Diodrast©)

The reversibility of unilateral barrier alteration induced by carotid injec tion of two acids of this type — acetrizoate (Urokon©) and penicillin G — was studied in the present long term experiments As these compounds also could elicit significant EEG changes in the injected hemisphere once the barrier was eliminated (GONSETTE 1956 FLODMARK and STEINWALL 1962 1963) they also served as tools in testing the state of the barrier by means of the EEG in early and late phases of the experiments The reversal of the barrier dysfunction was furthermore proved by means of administration of the two acid dyes Congo red and trypan blue soon after the carotid injection and at the end of the experiment respectively according to a technique introduced by BROMAN and OLSSON (1948) Both these dyes show a marked tissue affinity and a poor diffusibility which make them well suited for staining purposes but less apt to bring about neuronal (EEG) influences compared to other more easily diffusing acids like penicillin and Urokon

Methods

The experiments ere performed on adult rabbits in urethane anesthesia with a technique fully described in a previous paper (FLODMARK and STEINWALL 1962)

In principle the procedures included 1) EEG-recording throughout the whole ex periment 2) unilateral (left sided) blood brain barrier alterat on 3) indication of the current state of the barrier at different phases of the experiment

The EEG was recorded with bipolar technique by means of platinum plated electrodes connected to a transistorized Kaiser EEG machine For induction of the blood brain barrier alteration 13—14 % Urokon (sodium acetrizoate) or 4 % penicillin G was applied within the vessels of one hemisphere for about half a minute via the ipsilateral carotid artery The details of the procedures and the dosage of the administered indicator compounds are described in the type experiments below

Results

The observations in relation to the experimental procedures are presented in the following description of two experiments

Type experiment I (No 8 in Table I) The rabbit (2 2 kg) showed a symmetrical EEG pattern before and after catheterization of the left carotid artery (Fig 1 A) Via this catheter 13 % Urokon (5 ml) was perfused through the left hemisphere for 35 sec expelling the blood During the perfusion and the following 3 min EEG besides an arousal pattern showed depressed amplitudes on the injected side (Fig 1 B) When the EEG had regained symmetry (Fig 1 C) penicillin was administered intravenously in order to test the state of the barrier Two doses (0.2 g and 0 3 g) given within 15 min provoked only small and transitory EEG effects A third dose of 0 6 g given 45 min after the carotid injection was followed in the EEG within 5 min by a predominantly left sided spiking which persisted for several hours although with decreasing intensity (Fig 1 D_1 D_2 One hour after the carotid injection Congo red (1 % 14 ml) was injected i v for dye indication of the barrier defect During the following seven hours of the experiment the EEG changes gradually diminished *When the animal was in undisturbed anesthesia the pattern after* about 4 hours seemed rather symmetrical but stimulation by pinching still caused sharp potentials on the left side In the last phase of the experiment (about 8 hours after the carotid injection) another i v injection of 0 6 g penicillin was given This did not provoke any significant effects in the EEG during the remaining 30 min of the experiment (Fig 1 E F) Ten minutes before its termination trypan blue 1 % 15 ml was given i v The animal was exsanguinated and the brain vessels rinsed with 0 9 % saline The removed brain showed a distinct left sided red staining with no signs of trypan blue extravasation except in normally "non barred" regions (e g infundibulum and area postrema) which were definitely bluish

Type experiment II (No 5 in Table I) The rabbit (2 6 kg) was prepared as described before As barrier-damaging agent 4 % penicillin was applied via the left carotid artery for 30 sec (13 ml) By the end of this application marked EEG abnormalities were seen They started and dominated on the left side but also spread to the other hemisphere (Fig 2 B C) The high voltage sharp potentials were accompanied by motor twitching Congo red (1 % 14 ml) with sodium fluorescein (10 % 1 ml) was injected i v 6 min after the carotid perfusion The intense paroxysmal firing which started shortly after 2 B was

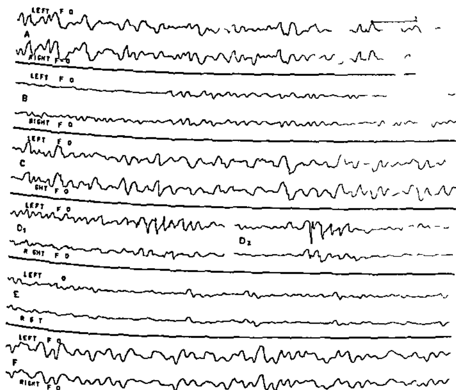


Fig 1 Type experiment I (no 8 in Table I) *EEG changes after intracarotid left-sided application of 13 % Urokon and subsequent intravenous loading with penicillin in the early and late phase of the experiment* (see text)

A Control record (urethane anesthesia) symmetrical pattern
B Three minutes after left-sided carotid perfusion of 13 % Urokon (5 ml) for 35 sec asymmetry with depressed amplitudes on the left (injected) side
C Four minutes after the carotid injection EEG symmetry regained
D Left-sided sharp potentials 5 min (D_1) and 1 hour (D_2) after the i v administration of 0 6 g penicillin.
E Seven hours after D A dose of 0 6 g penicillin was given 5 min before this record was made essentially symmetrical pattern (light sleep)
F Termination of the experiment, 8 1/2 hours after the carotid injection (about 30 min after E) essentially symmetrical EEG (medium sleep)
(F—O bipolar fronto-occipital leads)

recorded was still seen in the EEG 2 hours later (Fig 2 C) but within 5—6 hours the sharp potentials disappeared The remaining asymmetry gradually reversed but was still evident at the end of the 9 hour expt (Fig 2 D) Trypan blue (1 % 15 ml) was administered 10 min before the animal was sacrificed Exsanguination and rinsing of the vessels were carried out as before The removed brain showed Congo red staining of the left hemisphere while fluorescein (in ultraviolet illumination) was seen only in its posterior part The



Fig. 2. Type experiment II (no. 5 in Table I). *Prolonged EEG changes produced by the left-sided intracarotid perfusion of 4% penicillin in a long-term experiment.*
A. Control record (urethan anesthesia): symmetrical pattern.
B. Record 2 sec after intracarotid (left-sided) perfusion with 4% *penicillin 13 ml for 30 sec.* Sharp potentials predominantly on the left side.
C. Record 2 hours later. Intense paroxysmal firing from the left hemisphere starting a few minutes after I.
D. Record at the end of the 9 hour experiment. Persisting moderate asymmetry with slower waves on the left side. No sharp potentials.
(F—O: bipolar fronto-occipital lead.)

hemisphere showed no staining from trypan blue in contrast to the physiologically non-barred regions.

A summary of the experimental data appears in Table I. The occurrence of a predominantly unilateral barrier defect in the early phase was demonstrated by EEG activating tests in all experiments and further proved by staining with Congo red applied i.v. in this phase in 6 expts (no. 2, 5, 6, 8—10). The evoked EEG changes consisted of paroxysmal activity in all experiments but one (no. 2) in which the effect appeared as marked slowing of the pattern on the side of the carotid perfusion.

Restitution of the blood brain function was established in 6 animals (no. 5—10) as judged by the negative results of the dye test when trypan blue was given in the late phase of the experiments. This restitution was also indicated by the observation that penicillin injected i.v. in the same late phase produced no significant EEG changes (no. 7—10). In the remaining 4 expts a tendency towards restitution was suggested in 3 animals (no. 2—4). In expt no. 2 Congo red given in the early phase had stained the whole left hemisphere while the terminally applied trypan blue stained only a small area parietally. A corresponding reduction of the damaged region generating paroxysmal activity on EEG

Table I Schematic representation of the experimental data

Expt no	Barrier damaging agent perfused intracarotidally Urokon (U) Penicillin (Pc)			Results of barrier function tests				Duration of expt (hours)	Barrier restitution	EEG course
				Early phase		Late phase				
	Agent (conc)	Amount (ml)	Applic time (sec)	Dye test	EEG activating test	Dye test	EEG activating test			
1	U 14	9	35		+	+		4	?	Mainly left sided
2	U 13	9	30	+	+	(+)		8	Suggested by dye tests	changes provoked by the intracarotid
3	U 13	12	35		+	+	(+)	8	Suggested by EEG	injection and/or administra-
4	U 13	13	25		+	+	—	7	activating tests	tion of the acid compounds —
5	Pc 4	13	30	+	+	–		9	Proved by	tendency to nor-
6	Pc 4	10	30	+	+	—		8 5	dye tests	malization in
7	U 13	8	25		+	–	–	5	Proved by dye tests	the final phase of the experi-
8	U 13	5	35	+	+	—	—	9	and EEG	ment (concer-
9	Pc 4	14	18	+	+	—	–	6	activating	ning expt no
10	Pc 4	12	30	+	+	—	–	5 5	tests	3 see text)

activation in the early and late phases was observed in expt no 3 In expt no 4 the late phase dye test was slightly positive the EEG activating tests here suggested a tendency to recovery of the barrier function In experiment no 1 no information on barrier restitution was obtained

The course of the EEG in these long term experiments showed varying patterns as exemplified in the experiments to be described The injection of the barrier damaging agents influenced the EEG differently from a rather slight asymmetry up to a marked and protracted abnormality including paroxysmal activity In all cases these effects reversed in some experiments even to a symmetrical EEG pattern The EEG effects on the barrier damaged side after intravenous loading with acids also faded after various lengths of time (with the exception of expt no 3 where spiking from one part of the left hemisphere persisted until the termination of the experiment)

Discussion

The findings indicate that the barrier alteration caused by adequately dosed sodium acetrizoate (Urokon) and penicillin G is a reversible process as was the damage induced by the chemically related acid iodopyracet (Diodrast) according to BROMAN and OLSSON (1948) The nature of this reversible barrier

dysfunction is unknown. One possible way to imagine the process arises from the recently proposed hypothesis that the normal blood brain barring of organic polar compounds like the present acids at least partially is an effect of brain-blood directed transport mechanisms physiologically aimed for "excretion" of waste products (STEINWALL 1961, LIDSTRÖM and STEINWALL 1961). Such mechanisms might be blocked by overloading when exposed to too high concentrations of the polar compounds (i.e. the acids applied via the carotid artery in the present experiments), in a manner analogous to what is known to occur to the excretory transport apparatus in the renal tubules and in the liver. As in these organs the overloading effects on the barrier is thought to be reversible. During the state of overloading the acids do not meet the normal impediment to passage from the blood into the brain and thus they can be used for barrier damage indication when activating the EEG or staining the brain. After restitution of the transport function the barring effect is re-established; furthermore the already invaded acid compounds may be gradually transported from the brain if not too firmly bound to tissue components in a way which make them poorly available for transport. With respect to this transportability the 2 dyes Congo red and fluorescein can be expected to differ quantitatively according to the great difference in their tissue affinity. This dissimilarity might account for the observation reported in the description of expt. no. 5 (and also occuring in no. 6, 9 and 10) that fluorescein was found only in a part of the Congo red stained regions after termination of the experiment although the dyes were given together in the early phase. This phenomenon will be further analyzed in a separate study in our laboratory.

The EEG course in the present experiments with reversible barrier inhibition is different from that seen in the series with persistent barrier injury caused by mercuric ions (STEINWALL and FLODMARK 1963) where the electrical activity on the barrier-damaged side sooner or later became depressed and ultimately deteriorated. In the present series the barrier blocking carotid perfusion of the acid compounds, especially penicillin, could quickly give rise to marked ipsilateral EEG effects; these were, however, of an excitatory type and most likely exerted by surplus acid which could reach the nerve cells once the barrier impediment was eliminated. But even when the EEG changes evoked by this means or by additional intravenous loading with the acids were pronounced and of long duration, the final outcome was a clear tendency to normalization. The EEG influence of the 2 main indicator dyes, Congo red and trypan blue, was less apparent, which might be a consequence of their pronounced tissue affinity and poor diffusibility, rendering them less apt to reach the neuronal membranes.

The EEG abnormalities of excitatory (epileptogenic) type provoked by the acid compounds after blockage of the blood brain barrier in these experiment are comparable to effects observed both experimentally and clinically when other organic ions were applied topically (intrathecally) within the

central nervous system thus avoiding the barrier hindrance to their action on the neuronal membranes (McILWAIN 1957 CURTIS and ECCLES 1958 a b)

In forthcoming experiments other barrier damaging agents will be investigated with the aim to achieve optimal conditions for the study in the EEG of neuronal influences exerted by normally barred compounds administered in the general circulation

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Biochemical and Functional Effects of Long-Term Administration of Reserpine in Mice

By

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Abstract

MARKIEWICZ L. *Biochemical and functional effects of long term administration of reserpine in mice* Acta physiol scand 1963 58 376—380 — Mice were injected daily with reserpine (0 08 mg/kg) for 80 days Noradrenaline dopamine and 5-hydroxytryptamine were measured in brain noradrenaline in heart and adrenaline and noradrenaline in the adrenals The behaviour and the body weights of the mice were followed closely throughout the entire period of treatment The major part of the tissue monoamines disappeared during the initial period of the injections but there tended to be a recovery of the monoamines during the latter part of the experiment in spite of continued injections with reserpine The weight and the behaviour of the mice also showed the typical actions of reserpine during the initial period of injections (i e weight loss and sedation) but as the injections continued these functions also showed at least a partial recovery

In most of the work done so far with the aim of correlating biochemical and functional changes brought about by reserpine relatively large single doses of the drug have been used In this laboratory investigations have been started in which the long term effects of small daily doses of reserpine are studied HAGGENDAL and LINDQVIST (1962) have thus observed that in rabbits such treatment may lead to considerable depletion of catecholamines and 5 hydroxytryptamine (5 HT) in brain and other tissues without any gross disturbance of behaviour In the present paper a similar study in mice is reported

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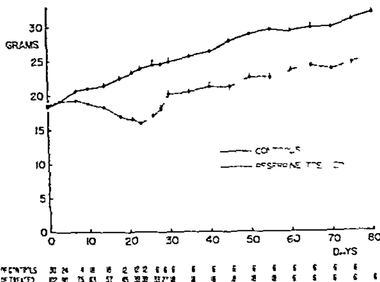


Fig 1 Effect of chronic reserpine treatment on weight of mice The mean weights ± S E are indicated The number of animals is shown below the graph.

Methods

The test mice were injected subcutaneously with reserpine 0 08 mg/kg/day in a 5 glucose solution Control mice were injected with an equivalent volume of the solvent Each day the mice were weighed and carefully observed throughout the day Six test or control mice were killed by decapitation 24 hours after the last injection The adrenaline and noradrenaline contents of the extracts of the tissues were determinated according to the method described by BERTLER, CARLSSON and ROSENGREN (1958 dopamine by CARLSSON and WALDECK (1958) modified according to CARLSSON and LINDQVIST (1962) and 5-hydroxytryptamine (5 HT) by BERTLER (1961) except that the perchloric acid residue was re-extracted

Results

Body weight and behaviour

After 7 days of treatment the body weights (Fig 1) of the reserpine treated mice started to decrease After 3 weeks the body weights started to increase again and continued to do so throughout the experiment although they remained at a lower level than the weights of the control animals

During the first 5 days there were no marked differences between the behaviour of the test and control mice The only noticeable effect of reserpine (aside from the loss of weight) was a ptosis which occurred already after the first injection After 5 days the mice were sedated following each injection and this effect tended to persist for a longer duration with increasing injections Gradually anorexia appeared but on about the 20th day of treatment the

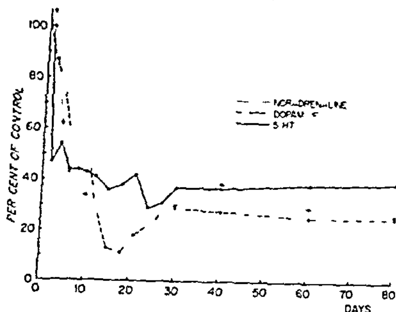


Fig 2 Effect of chronic reserpine treatment on 5-hydroxytryptamine noradrenaline and dopamine content in brain

started to eat more However even after 23 days the mice looked poor and the weights of the experimental mice had decreased to 67 per cent of the controls After 4 weeks however the test mice could not be distinguished from the controls except for a period of sedation lasting approximately 6 hours after the injection They remained in this condition until the end of the experiment

Effect of reserpine on the concentration of monoamines in brain

The concentration of 5 HT in mouse brain decreased to about 30 per cent of the control value in about 2 weeks and remained at this level for the rest of the experimental period The noradrenaline level showed a similar course (Fig 2)

The dopamine decreased to about 10 per cent of the control value in about 2 weeks and then seemed to rise again up to about 30 per cent in the following 2 weeks. This level was maintained for the rest of the experiment

Effect of reserpine on catechol amine levels in extracerebral tissues

The noradrenaline content in heart declined more rapidly than in brain a decrease of about 50 per cent occurring within 24 hours After 4 days the noradrenaline had decreased to about 10 per cent and remained at a low level for the rest of the experiment (Fig 3)

The adrenaline and noradrenaline of the adrenal glands decreased to about 30 and 15 per cent respectively, during the first week The results are expressed

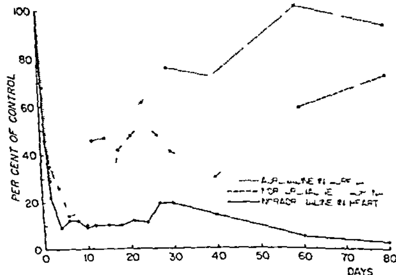


Fig 3 Effect of chronic reserpine treatment on noradrenaline content in heart and on noradrenaline and adrenaline contents in adrenals

as μg of amine per pair of adrenals because reserpine causes an increase of the weight of cortex (KHAZAN SULMAN and WINNIK 1961) During the following 2 weeks the noradrenaline concentration increased to about half the control values but then decreased again during the subsequent 2 weeks (between the 24th and the 40th day) It then slowly increased again The adrenaline level started to rise on the 10th day In contrast to noradrenaline the rise in adrenaline was continuous Towards the end of the experiment the adrenaline level was nearly normal

Discussion

In these experiments 2 periods can be distinguished The former period which lasted a couple of weeks is characterized by cumulation of both functional and biochemical effects The latter period is characterized by marked though not complete functional recovery Biochemically the recovery was less complete in general but varied in different tissues and for different amines In brain a definite though partial recovery of dopamine only was seen In heart noradrenaline showed no recovery The most clearcut recovery of amine levels was seen in the adrenals where particularly adrenaline returned to nearly normal values The adrenal noradrenaline showed a two-phase recovery which may be related to the transient rise in adrenal noradrenaline observed during recovery from e g a single dose of reserpine by CALLINGHAM and MANN (1962)

The considerably more marked recovery of the adrenal amines as compared with the amines of the other tissues may be related to the well known fact that the action of reserpine on the adrenal medulla is partly centrally mediated The anomalous behaviour of the adrenals in the present experiments may therefore be secondary to the functional recovery of the brain

The explanation of the functional recovery observed in the present experiments in spite of continued dosage of reserpine and low monoamine levels in brain must await further studies It seems possible that the monoamine storage mechanism showed a higher degree of recovery than the level of monoamine stores Cellular adaptation according to the "law of denervation is another factor to consider

I wish to express my deep gratitude to Professor ARVID CARLSSON for his advice and continued interest throughout this work I am indebted to the Swedish Ciba Ltd Stockholm for generous supplies of Serpasil

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Function of the Corneal Nipples in the Compound Eyes of Insects

By

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The compound eyes of certain insects contain on their corneal surfaces a hexagonal array of nipples the dimensions of which are about 200 mμ base to tip and center to center (BERNHARD and MILLER 1962) The following investigations were carried out in order to elucidate the functions of these nipples

1 *Spectrophotometric measurements* Spectral transmission of insect corneas was measured between 700 mμ and 230 mμ All corneas investigated show little change in density for the visible spectrum but have a sharp increase in density in the ultraviolet with a peak at 275 mμ which was shown to be a property of corneal chitin and unrelated to the presence of nipples

2 *Microwave experiments* Appropriately scaled dielectric models of insect corneas were cast using various waxes that have values for the square root of the dielectric constant that approximate the index of refraction of corneal chitin Measurements of the *transmission* through these model before and after removal of the nipples show that the nipples cause an increase in optical density as measured on the optical axis There is a peaking of optical density of about 1 5 log units where the wavelength (λ) is half the nipple height (d) i e λ/d = 0 5 Measurements made off the optical axis show that this increase in density is the result of a redistribution of energy and hence it is assumed to be a manifestation of an interference phenomenon caused by the hexagonal array of nipples For values of λ/d of 1 5 to 5 0 the presence of nipples results in a small increase in the amount of transmitted energy Transmission through the models is not a function of the plane of polarization of the microwaves *Reflection* from the surfaces of models before and after removal of the nipples was also investigated The presence of nipples results in decreased reflection of about 0 5 log units over a broad range of values of λ/d Experiments on metal models show that the reflection is independent of the presence of metal nipples Hence, *change in reflection due to the presence of nipples* is not caused by scatter

When applied to the insect cornea the results of the transmission experiments on the models indicate that the density increase at λ/d = 0 5 would occur in a region of the ultraviolet part of the spectrum where almost no energy reaches the surface of the earth Therefore this particular phenomenon is thus of have no biological significance However throughout the visible spectrum and near ultraviolet the microwave experiments indicate that the nipples r

marked decrease in reflection from the front surface of the cornea and a corresponding increase in transmission through the cornea

3 *Light microscope investigations* Experiments in which light reflected from insect corneas floating on india ink was photographed confirm the observations made on models that the presence of *nipples decreases the light reflection from* the facet surface The nipples decrease the reflection by something less than a log unit over a broad range of wavelengths including the Hg I line at 365 *mu* but do so less effectively for the longest visible wavelengths The nipples do not impair the transparency of the cornea

Conclusions The nipples found on the corneal surface decrease reflected energy from the surface and correspondingly increase transmission through the cornea In this respect the nipple array resembles man made antireflection coatings

The mechanisms of this natural coating may be understood in terms of theories *generally applied in the study* of artificial coatings in optics An optimal antireflection coating would be achieved by making a gradual transition between the index of refraction of air and that of the lens material Because this cannot be conveniently realized by the application of homogeneous coatings a practical approximation to the ideal is achieved by covering the lens surface with a single layer of homogeneous transparent material of low refractive index *For wavelengths close to four times the thickness of the* coating reflection will be substantially diminished However for coatings that make a gradual transition theory predicts that the reflection will be diminished over a broad range of wavelengths (BLAISSE 1950 see also STRONG 1950) the thickness of the coating is therefore not critical but should be about half a wavelength for the middle of the spectrum concerned Evidently the natural type of coating found on insect corneas achieves the theoretical optimal conditions for the most effective antireflection coating The nipples are small in relation to wavelength of visible light Therefore the nipples and the air spaces between them have the appearance of homogeneous material which provides a gradual increase in density thus giving a smooth transition of refractive index from that of air to that of the cornea

By diminishing reflection from the corneal surface the nipples improve camouflage without impairing visual acuity In fact there is a slight increase in light transmission All of these factors are of importance particularly for nocturnal insects

These investigations were supported in part by the United States Air *Force under* Grant No AF — EOAR 62—13 and monitored by the European Office of Aerospace Research (C G Bernhard principal investigator) and in part by Research Grant B-864 from the National Institute of Neurological Diseases and Blindness United States *Public Health Service* B (H K Hartline principal investigator) The research has also been supported by the Swedish *Medical Research* Council The Department of Medical Physics and the Department of Medical Cell Research and Genetics at Karolinska Institutet have made available *to us their* equipment and technical facilities We are particularly indebted to Professor B Agdur of the Microwave Department at the Royal Institute of Technology Stockholm for his advice and for his generosity with his equipment

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Cellular Localization of Monoamines in the Median Eminence and in the Infundibular Stem of Some Mammals

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Monoamines probably catecholamines have been found accumulated in the median eminence with the help of a histochemical fluorescence method (CARLSSON FALCK and HILLARP 1962) However their cellular localization could not be decided on The purpose of the present investigation was to determine in what cell structures the amines are localized and their relation to the portal system

The median eminence and the infundibular stem of the mouse rat hamster guinea pig rabbit and cat were freeze-dried treated with formaldehyde gas embedded in paraffine sectioned and mounted for fluorescence microscopy according to FALCK (1962) It was found that the cellular localization of the monoamines was much more easily determined in certain species, namely the cat and the guinea pig In the median eminence and the infundibular stem of these species the fluoresescent material was observed to be localized within fine nerve fibres which are concentrated in the superficial zone very closely surrounding the primary capillary plexus which is drained into the portal vessels In cross sections the fluorescent nerve fibres form an intensely green to yellow green fluorescent conture which surrounds the central zone which contains much less fluorescent fibres than the superficial one The fibres have the characteristic appearance of terminal nerve fibres (cf HILLARP 1959 FALCK 1962) with typical varicosities exhibiting an intense fluorescence Noradrenaline has been demonstrated within hypothalamic synaptic terminals of this appearance (CARLSSON et al 1962)

Since the tubero-infundibular system ends in the same region (cf SZENTAGOTHAI et al 1962 as the observed nerve endings aminergic neurons were searched for in the region where the tubero-infundibular tract system arises

Small nerve cells with a weak green fluorescence were found to be situated in *the arcuate nucleus and the ventral portion of the anterior periventricular* nucleus (see also CARLSSON *et al* 1962)

The specific green to yellow green fluorescence in the fibres and in the nerve cell bodies disappeared in all species after reserpine treatment In the rat a large decrease of the fluorescence was observed after a dose of only 0.5 mg/kg (24 hr) It had disappeared in *mice and rats which were killed 2 hr after i p* administration of m tyrosine (400 mg/kg, 3 times with 2 hr intervals) Since the histochemical criteria (see FALCK 1962) also were fulfilled there seems to be little doubt that the fluorescent material is derived from primary catechol amines Dopamine may well be the chief amine present in the fluorescent nerve fibres surrounding the primary capillary plexus since fluorescence could be developed in most of them 24 hr after administration of *a* methyl m tyrosine (400 mg/kg i p to mouse rat and guinea pig) (*cf* CARLSSON *et al* 1962)

The fluorescent terminals were still present 2 to 17 days after cervical sympathectomy (rat and guinea pig) They thus probably belong to central neurons and may originate from the catecholamine containing nerve cells detected in the region where the tubero-infundibular tract arises

The finding that catecholamines are accumulated in high concentrations in nerve terminals surrounding the primary capillary plexus of the portal system indicates that these amines act as neuro-humoral transmitters for the regulation of the activity of the anterior pituitary

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